Low Emission Feed

Opportunities to mitigate enteric methane production of dairy cows

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PREFACE

The work described in this thesis is based on experiments conducted at the Animal Nutrition group, Wageningen University. A research project was developed to investigate the effects of feeding strategies on enteric methane production of dairy cows. The experiments were conducted at the Animal Research Facilities of Wageningen University (de Ossekampen and Carus), and in the laboratory of the Animal Nutrition group.

In this thesis, the results described are from research work that investigated the effects of types and levels of starch in the concentrate, different quality of maize silage and grass silage, and condensed tannins on methane production of dairy cows. Experiments were conducted by feeding different types and levels of starch in the concentrate, different quality of maize silage and grass silage to dairy cows and measurement of the daily emissions of enteric methane from individual cows using climate-controlled respiration chambers. In relation to the types and levels of starch in concentrate, an in vitro experiment alongside in vivo experiment was conducted to make a comparison between in vitro and in vivo methane production of dairy cows measured simultaneously, using the same cows as donor for microbial inocula when they were fed and adapted to the same dietary material used as a substrate for in vitro incubation. Experiments with regard to the condensed tannins were based on in vitro studies. In six chapters and a general discussion, different parts of the research are described and discussed.

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ABSTRACT

As global demand for high-quality food originating from animal production is expected to rise due to an increasing human population and consumer income level, the expected role of ruminants in meeting this demand brings multiple challenges. Ruminant production needs to adapt to environmental changes and, at the same time, reduce its impact on the environment. Ruminants production systems have a major impact on the environment through the emission of greenhouse gases such as methane (CH_4), nitrous oxide and carbon dioxide. Microbial fermentation of feeds in the gastrointestinal tract, known as enteric fermentation, is the main source of CH_4 emissions from dairy production. Enteric CH_4 emission is strongly related to the amount of feed fermented in the rumen, which depends on feed intake, feed composition and rumen fermentation conditions associated to the intrinsic characteristics of these feeds and the characteristics of the whole diet. Important gaps in knowledge remain however. The prime aim of this thesis was to investigate the effects of various feeding strategies to mitigate enteric CH_4 emissions of dairy cows.

First experiment was conducted to investigate the effects of type and level of starch in the concentrate. Inclusion of a high level (53%) of starch in the concentrate that accounted for 40% of the total mixed ration dry matter (DM) produced lower CH_4 per unit of estimated rumen fermentable organic matter (eRFOM) than a low level (27% of DM) of starch (43.1 vs. 46.9 g/kg of eRFOM). Methane production per kg of eRFOM also was lower for diets based on rapidly fermentable starch (gelatinized maize grain) compared to diets based on slowly fermentable starch (native maize grain) (42.6 vs. 47.4 g/kg of eRFOM). However, inclusion of 53% of starch in the concentrate from both types of starch did not affect CH_4 emission intensity (CH_4 Ei) (CH_4 emission per kg of fat- and protein-corrected milk; FPCM). In a subsequent experiment, maize silage was prepared from whole-plant maize harvested at a very early (25% DM), early (28% DM), medium (32% DM) and late (40% DM) stage of maturity and fed to dairy cows as an alternative to concentrate as starch source. Diet consisted of (on DM basis) 75% maize silage, 20% concentrate and 5% wheat straw. Increasing harvest maturity of maize silage linearly decreased CH_4 yield (21.7, 23.0, 21.0 and 20.1 g/kg of DM intake) and CH_4 emission as a fraction of gross energy intake (6.3, 6.7, 6.3 and 6.0%). Methane Ei tended to decrease linearly with maturity (13.0, 13.4, 13.2 and 12.1 g/kg FPCM). In another experiment grass silage as roughage source was tested. This experiment was designed to investigate the effects of N fertilisation of grassland and maturity of grass at cutting on CH_4 emission in dairy cows. Two N fertilisation rates (65 vs. 150 kg of N/ha) were examined in combination with three stages of grass maturity (early, 28 days of regrowth; mid, 41 days of regrowth; and late, 62 days of regrowth). Diet contained 80:20 ratio (on DM basis) of grass silage (mainly ryegrass) and concentrate. Dry matter intake decreased with N

fertilisation and maturity, and FPCM decreased with maturity but was unaffected by N fertilisation. Methane Ei (mean 15.0 g/kg of FPCM) increased by 31% and CH_4 per unit digestible OM intake (mean 33.1 g/kg of DOMI) increased by 15% with increasing maturity. Methane yield (mean 23.5 g/kg of DM intake) and CH_4 as a fraction of gross energy intake (mean 7%) increased by 7 and 9% with maturity, respectively, which implies an increased loss of dietary energy with progressing grass maturity. Rate of N fertilisation had no effect on CH_4 Ei and CH_4 yield.

Despite the importance of in vitro gas production technique for evaluating feeds, in vitro study as a stand-alone approach was considered inadequate to fully evaluate the potential effect of feeds and rumen fermentation modifiers on CH_4 production, because in vitro studies are frequently performed separately rather than in parallel with in vivo studies. To test this hypothesis, both in vitro and in vivo CH_4 measurements were measured simultaneously using cows in the first experiment that were fed (and adapted to) the same dietary material used as a substrate for in vitro incubation, as donor for microbial inoculum. It was found that 24-h in vitro CH_4 (mL/g of incubated organic matter) correlated well with in vivo CH_4 when expressed per unit of eRFOM (R^2 = 0.54), but not when expressed per unit of organic matter ingested (R^2 = 0.04). In the same experiment, results showed that incubation of the same substrate with rumen inocula obtained from donor cows adapted to different diets produced a variable amount of CH_4 suggesting that it is important to consider the diet of the donor animal when collecting rumen inocula for in vitro incubation. Even though the in vitro technique has limitations to represent in vivo conditions, it is useful for screening of large sets of animal feeds or feed additives to be used as a CH_4 mitigation strategy. In this thesis, two in vitro experiments were conducted to examine the effects of variation in structural composition of condensed tannins (CT) in sainfoin accessions collected from across the world on CH_4 production, and CT extracts obtained from a selected sainfoin accessions on CH_4 production. Results revealed substantial variation among CT in their effect on in vitro CH_4 production and this variation was attributed to differences in chemical structure of CT. Condensed tannins evaluated in this thesis showed to have potential to reduce in vitro CH_4 production, but require further investigations to fully evaluate their in vivo effects.

In conclusion, results from the research work conducted in this thesis show that changes in the basal diet of dairy cows and in roughage production management can substantially reduce the amount of enteric CH_4 produced and thereby influence the impact of dairy production on the environment.

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CHAPTER 1

General introduction

The rumen harbours a complex and highly diversified microbial population comprising mainly of bacteria, protozoa and fungi. The rumen microbes reside symbiotically with the host and grow through the normal process of microbial fermentation of feeds ingested by the animal, called enteric fermentation. By this process, large amounts of coarse plant materials that are largely indigestible to monogastric animals including humans are degraded and produce microbial biomass, volatile fatty acids (VFA; mainly acetate, propionate and butyrate), hydrogen, methane (CH_4) and carbon dioxide (CO_2) as the main end-products. After VFA are absorbed through the rumen wall into the circulatory system, they are utilized by the host and supply a major part of the energy needs of the host. The microbial cells, together with undegraded feed components, pass to the small intestine to be digested by host enzymes supplying protein to the host. This adaptation of ruminants to derive much of their nutrient supply from cellulose and hemicellulose has enabled them with a unique ability in maintaining and enhancing the supply of high-quality protein sources such as meat and milk and micronutrients from human inedible feeds. This makes ruminant animals not to be considered as obligated-competitors with humans for food resources. Even when some competition could occur, like feeding of grains, ruminants in general are necessary to produce high quality protein in milk and meat for human consumption.

The projected human population growth and rising incomes are expected to drive the consumption of meat and milk, and global demand for these animal products is projected to be more than double by 2050 compared to 2000 (Steinfeld et al., 2006; FAO, 2011). As global demand for animal proteins continues to rise, ruminant production faces multiple challenges. Ruminant production needs to supply high-quality foods to meet the expected demand of an increasing human population, adaptation to environmental change and, at the same time, reduce impact on the environment. The impact of livestock production on the environment includes emission of greenhouse gases (GHG) such as CH_4, nitrous oxide and CO_2 either directly (enteric fermentation, stored manure, feed production activities) or indirectly (land use change such as conversion of forest into pasture land, ammonia emissions from applied manure, fertilizer and stalls, and nitrate leaching from soils). Enteric fermentation is the main process contributing to direct non-CO_2 GHG emissions from dairy production and is the largest source of CH_4 next to natural sources (wetlands, oceans, lakes, rivers and termites) (Knapp et al., 2014). Methane production by livestock accounts for approximately 14.5% of global GHG emissions (Gerber et al., 2013).

Research on reducing enteric CH_4 production from ruminants with the prime aim of achieving increased feed conversion ratios already started during the 1960s (Blaxter and Clappert, 1965). However, as concerns regarding climate changes grew and pressure to reduce GHG emissions increased in the last decades, it received renewed research attention. The increase in emission of GHG including CH_4 from ruminant livestock production enforces industrialised countries to reduce their GHG emissions and signed a treaty namely the Kyoto protocol in December 1997. The Kyoto protocol is a legally binding agreement under which industrialized countries including the European Union agreed to reduce their collective emissions of GHG by an average of 5.2% against 1990 level over the five-year period 2008-2012. As follow-up of this negotiation, the EU went further and promised 20% reduction relative to 1990 levels (European Commission, 2010) by 2020. Some countries such as the Netherlands have committed to 30% reduction. Achieving this target forms a major challenge for governments and requires exploration of strategies to mitigate CH_4 emission from dairy production. If a strategy that reduces CH_4 production coincides with a higher energy supply to the animal, it will support achieving the targets for the Kyoto protocol and decrease the carbon footprint of animal product. In order to explore strategies to reduce enteric CH_4 production, it is important to understand how fermentation processes are controlled in the rumen.

Complex carbohydrates (including cellulose, hemicellulose, pectin, starch and sucrose) ingested by ruminants are oxidized to pyruvate, 3-carbon simple sugar, as an intermediate product by microbes residing in the rumen. The pyruvate thus generated is transformed into different fermentation end-products through multiple-steps of metabolic pathways that produce metabolic hydrogen, reducing equivalents (Moss et al., 2000; McDonald et al., 2002). The fermentation of pyruvate involves oxidation reactions under anaerobic conditions producing reduced co-factors such as NADH. This reduced co-factor is then re-oxidized to NAD to complete the synthesis of VFA. As described by McDonald et al. (2002) and Moss et al. (2000), the main anaerobic fermentation pathway of pyruvate to acetate and butyrate produces hydrogen, whereas production of propionate utilizes metabolic hydrogen. The hydrogen produced during enteric fermentation is utilized by a group of methanogens residing in the rumen and known collectively as archaea to reduce CO_2 to CH_4 (McAllister and Newbold, 2008). Methanogenesis is the major route of eliminating hydrogen produced in the rumen. The reaction that involves the reduction of CO_2 to CH_4 through utilization of hydrogen is thermodynamically favourable to methanogens to generate metabolic energy in the form of

ATP that is subsequently utilized for their maintenance and growth (Ellis et al., 2008). By utilizing hydrogen produced as a terminal step of carbohydrate fermentation, methanogens play a key role in rumen fermentation by maintaining a low concentration of hydrogen in the rumen and allowing the microorganisms involved to function optimally and support the continuation of substrate fermentation. If hydrogen is not removed from the rumen, it inhibits the re-oxidation of NADH, microbial growth and fibre degradation (Sharp et al., 1998; McAllister and Newbold, 2008). However, the CH_4 produced by the ruminal methanogens is released to the atmosphere by the ruminants, through breath and eructation, and is considered as a driver of global climate change (Wuebbles and Hayhoe, 2002).

Climate change is becoming a worldwide concern because of its significant and negative impacts on people, natural resources, and economic conditions around the globe (Abbasi and Abbasi, 2010). Though ruminants enhance the supply of high quality protein sources for human consumption and the formation of CH_4 benefits many microbes in the rumen, contributing to the efforts to reduce global warming by reducing enteric CH_4 emissions from dairy cows will play also a vital role in contributing to the solutions to climate change obligations. Methane has a global warming potential (GWP) of 25 times to that of CO_2 (IPCC, 2007). Due to this high GWP and relatively short atmospheric lifetime, it is more important to target reduction of CH_4 emissions over strategies focusing on reduction of CO_2 emission alone; because of the large effect that CH_4 reduction can have within the short-term if no significant reduction of CH_4 is achieved. In addition, enteric CH_4 production represents a loss of energy to the animal. According to the IPCC (1997), an average 6.5% of gross energy (GE) ingested by dairy cows is lost as CH_4, which is not available for growth or milk production. This energy loss can vary between 2 and 12% depending upon the quality of the diet (Johnson and Johnson, 1995). With such a significant impact, it is very important to investigate strategies available to mitigate enteric CH_4 emission from dairy production.

Textbox 1.
The capacity of GHG to trap heat in the atmosphere is described in terms of their GWP, which compares their atmosphere warming potential to that of CO_2 (with a GWP set at 1). In other words, the GWP indicates the amount of heat trapped per mass of gas and the time the gas remains in the atmosphere.

STRATEGIES TO MITIGATE ENTERIC METHANE PRODUCTION OF RUMINANTS

For the past decades, much research was directed at finding or testing strategies to reduce enteric CH_4 emission. Although a great deal of information has been generated from these

studies (Moss et al., 2000; Boadi et al., 2004; Beauchemin et al., 2008; Martin et al., 2010), there is more research to be done in this field. Possible mitigation options or strategies can be broadly grouped into three main categories: 1) manipulation of the animal itself, 2) diet manipulation and 3) modifications of the rumen microbial population (Figure 1). These strategies have varying degrees of feasibility, efficacy, persistency, and some are still hypothetical or only have been demonstrated in vitro and need to be confirmed in vivo. Some of these mitigation strategies may also have a short-lived effect due to adaptation of the rumen

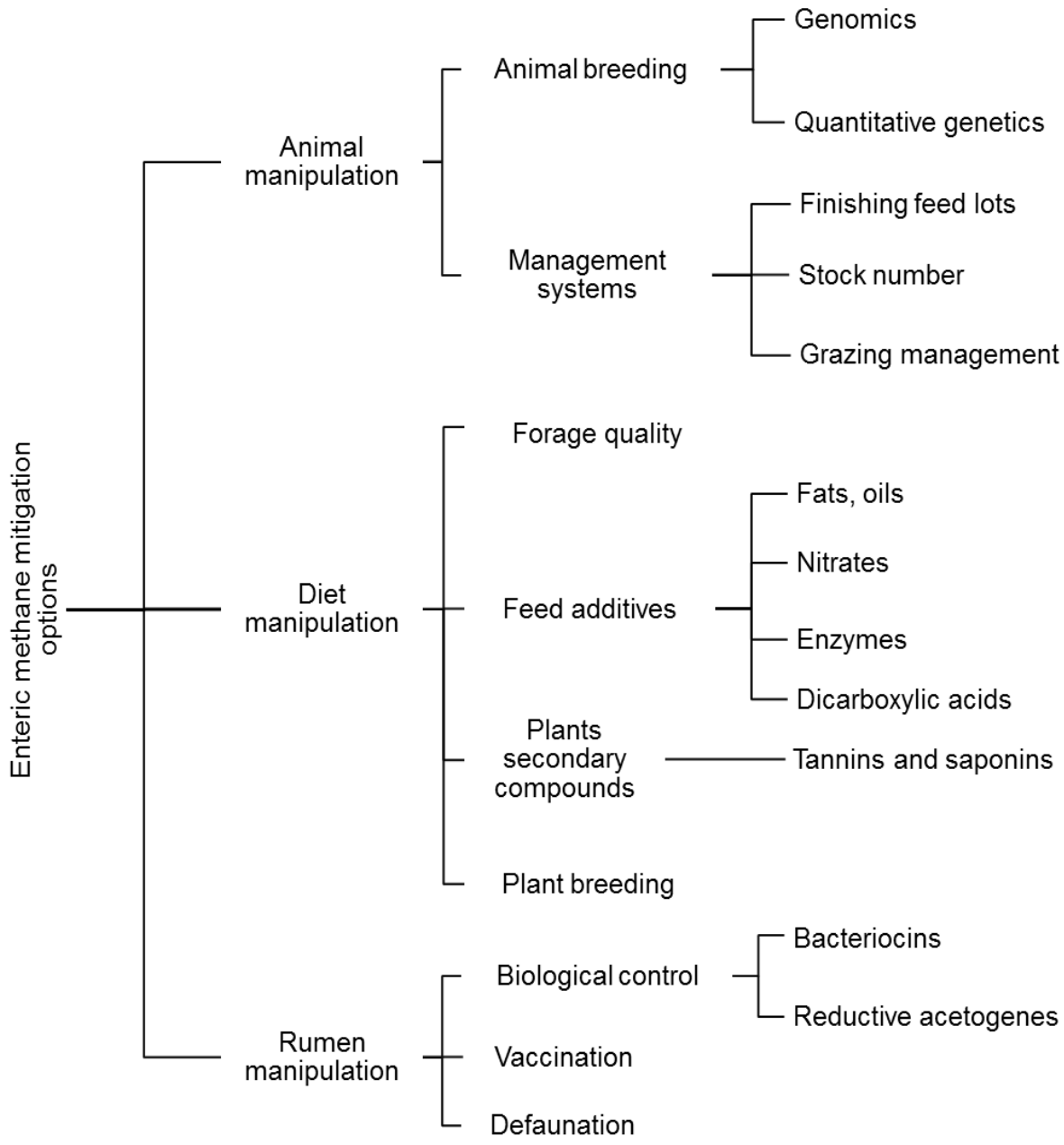

Figure 1. Overview of conceptual strategies to mitigate enteric CH_4 production from ruminants (adapted from Eckard et al., 2010 and Cottle et al., 2011).

microbial population (Hook et al., 2009), or are prohibited from use (e.g., monensin in the EU), are excessive in cost or have variable effects in vivo (such as organic acids, e.g. fumarate) (McGinn et al., 2004; Molano et al., 2008; Wood et al., 2009), have a negative effect on quality of animal product, e.g. milk fat depression (Bauman and Griinari, 2003), potentially toxic to the rumen microbial community (Ungerfeld et al., 2004; Guo et al., 2007), or reduce feed digestibility (Goel and Makkar, 2012).

Identifying strategies that can reduce CH_4 production without negative consequences on feed digestion and animal productivity are a significant challenge. It is of main scientific interest to investigate potential mitigation strategies to reduce CH_4 emissions from dairy production. Enteric CH_4 emissions correlate positively to the amount of dry matter (DM) fermented in the rumen, which itself depends on feed composition and rumen fermentation characteristics of the diets (Beauchemin et al., 2008; Hristov et al., 2013). Still large gaps in knowledge are present which require substantial research. The effect of feeds on enteric CH_4 reduction could be due to a direct (suppression of microbial activity), or an indirect impact on microbial fermentation of feed and lead to different proportions of end-products that are not equivalent in terms of hydrogen produced (Bannink et al., 2011). For instance, both cellulose and starch are hydrolyzed to glucose in the rumen, which is catabolized through glycolysis to pyruvate (Janssen, 2010). When glucose is released from cellulose digestion, pyruvate is prefertially metabolized to acetate, along with CO_2 and hydrogen. But, when glucose originates from starch metabolism, more propoinate is produced from pyruvate which decreases the concentration of hydrogen in the rumen (Bannink et al., 2006). The higher propionate production competes with methanogenesis for reducing equivalents and less CH_4 per unit of feed fermented is expected. Hence, strategies that can change the rumen fermentation pathways that lead to a relatively higher yield of propionate lowers net hydrogen production (Hegarty, 1999). Generally, if the composition of the feed determines the products, then the different amounts of CH_4 formed per unit of feed fermented can easily be explained. However, this is not always true, as there is a certain level of metabolic flexibility in the rumen, allowing different combinations of substrate utilization and fermentation pathways to co-exist (Jansen, 2010).

Although roughages are the main part of the diet of ruminants, consequences of management options to improve roughages quality (such as digestibility and nutrient composition) on CH_4 emission are not well documented. Furthermore, effects of type and quantity of starch in the diet have not been well documented despite of the high importance of

starch for example during post-calving lactation. In order to mitigate CH_4 emissions via modification of roughages and concentrate composition from dairy cows, it is important to investigate and quantify the effects of these feeding options on enteric CH_4 production. Modifying the diet (option two; Figure 1) thus has an advantage that can be applied immediately by the end-users and is among the most feasible option. Therefore, research work in this thesis mainly focuses on three areas: 1) investigating the source and level of starch in the diet, 2) examining the effects of improving quality of grass silage through grassland management, and 3) investigating the potential of plants containing condensed tannins (CT).

1. Dietary starch sources

Starch is the most common non-fibre carbohydrate (NFC) energy source for high-producing dairy cows. Starch in ruminant diets is mainly supplied by concentrate using cereal grains such as maize, wheat, barley and oats, and by forage crops such as whole-plant maize and wheat silages. However, the cereal grains differ in their starch content, with wheat containing (on DM basis) 77% starch, maize 72%, and barley and oats 58% (Huntington, 1997). In addition, differences in the chemical and physical structure of starch granules determine the quality of starch in feed and its degradability. Earlier studies comparing the rates of starch fermentation of five cereal grains using in vitro and in situ techniques showed that when cereal grains were processed similarly, oats fermented more rapidly than wheat, followed by barley, maize, and finally sorghum (Herrerasaldana et al., 1990). Between 80 and 90% of barley and wheat starch, and 92 to 94% of oats starch is degraded in the rumen, whereas the values for maize starch range from 55 to 70% (Nocek and Tamminga, 1991; Huntington, 1997). Microbes rapidly digest the protein matrix in barley grain, which embeds the starch (McAllister et al., 1993), whereas the protein matrix of maize grain is more resistant to digestion by ruminal bacterial amylase (Mills et al., 1999). Relative to barley, a substantial fraction of potentially fermentable maize starch may reach the small intestine unfermented, and may be digested enzymatically to glucose in the small intestine adding to the energy supply of the animal without associated losses of energy with CH_4 production (Dijkstra et al., 2011).

Increasing the starch content in ruminants' diet is suggested as an effective way of decreasing CH_4 emission intensity (CH_4 Ei; CH_4 per unit of animal product) (Hristov et al., 2013). In addition, the proportion of NDF versus NFC in the ration (Sutton et al., 2003; Bannink et al., 2006) influences the relative proportion of VFA produced in the rumen. However, studies relating the effect of sources and levels of starch in the concentrate to the

quantity of CH_4 produced are limited, and starch sources have seldom been tested in a systematic manner and under well-controlled conditions in vivo. This could be of interest as there is a large selection of concentrate ingredients available, ranging from cereals (low in fibre, high in starch) to cereal by-products (high in fibre, low in starch) or pulps (high fibre). Research is required to establish if altering the type and level of starch in the concentrates can significantly affect enteric CH_4 production of dairy cows.

Next to the fermentation characteristics and level of starch in the concentrates, also the quality and quantity of starch from roughage may affect enteric CH_4 production. Whole-plant maize silage is the most important cereal roughage and a major energy source in dairy cattle rations in many parts of the world. Starch content of maize silage is an important factor affecting its nutritional quality. Ensiled maize, with a relatively high starch content and a high digestibility has been reported to improve animal performance relative to grass silage (Mayne and O'Kiely, 2005) with a potential benefit of a reduced CH_4 Ei (Beauchemin et al., 2008), and its ruminal starch fermentation may give more propionate and hence less CH_4 than sugars and fibre (Bannink et al., 2006). However, the starch content of maize silage is mainly affected by the stage of maturity of the plant at harvest (Johnson et al., 1999). Although the importance of changes in the nutritional components of maize silage made from crops harvested at different stages of maturity have been well investigated with respect to nutritional and production responses by ruminants livestock in numerous studies (see recent review of Khan et al., 2015), limited studies are available which systematically evaluated the effects of maturity of maize at harvest on CH_4 emissions of dairy cows fed maize silage. The changes in nutritional composition of maize with maturity is expected to decrease CH_4 Ei of cows fed silage made from maize harvested at a late compared with an early stage of maturity, and this formed the basis for the design of this experiment.

2. Improving quality of grass silage

In addition to the type of starch in the concentrate and starch from maize silage, improving the quality of grass silage through grassland management is usually anticipated to lower CH_4 Ei as well, since it enhances animal productivity (Hristov et al., 2013). Improving grass silage digestibility and energy content, and better matching protein supply to the animal's requirements can be achieved through better grassland management. These measures can improve nutrient uptake and increase animal productivity, and thus lower CH_4 Ei or per unit of digested organic matter (OM) (Brask et al., 2013). Grass, being either grazed or used as silage, is economically the most accepted forage to supply energy and protein to ruminants.

Grass silage is the main component of dairy cow rations in the Netherlands (Tamminga et al., 2007) and in many other parts of the world. About half of the ration of dairy cattle in the Netherlands consists of grass, either fresh or ensiled with grass silage by far the largest component. Management factors such as nitrogen (N) fertilisation rate and the maturity of grass at harvest influence rumen degradation characteristics (Heeren et al., 2014), chemical composition (sugars, protein and fibre contents), digestibility and nutritional value (Warner et al., 2015). Digestibility of fibre (cellulose and hemicellulose) was shown to have a strong relationship with CH_4 production (Holter and Young, 1992). Earlier, Moe and Tyrrell (1979) predicted that for every gram of cellulose digested, CH_4 emission is nearly three times that of hemicellulose and five times that of the soluble residue. A study by Boadi and Wittenberg (2002) also demonstrated that a reduction in forage in vitro OM digestibility led to an increase in gross energy ingested lost as CH_4. A simulation study with a mechanistic model (Bannink et al., 2010) showed there is a large effect of changing grass characteristics (chemical composition and in situ ruminal degradation characteristics) on simulated CH_4 production. However, carefully designed in vivo studies that evaluated the effects of grassland management strategies such as level of N fertilisation and maturity of grass at harvest, as well as their interaction on CH_4 emission by dairy cows are not available and requires further investigation.

3. Use of condensed tannin containing plants

The use of plants containing naturally occurring secondary compounds such as condensed tannins (CT) in dairy cattle nutrition has been reported to have anti-methanogenic properties (Beauchemin et al., 2007; Martin et al., 2010). Tannins bind with feed or microbial proteins forming tannin-protein complexes, which reduce degradation of plant protein in the rumen, thereby enhancing the flow of feed protein to the intestines or inhibit the activity of rumen microbes (Min et al., 2003). Condensed tannins of various origins have been shown to inhibit ruminal CH_4 production either when fed to ruminants as tannin-containing forages (Woodward et al., 2001; Puchala et al., 2005) or as tannin extracts tested in vitro (Pellikaan et al., 2011; Hassanat and Benchaar, 2013) or in vivo (Beauchemin et al., 2007; Bhatta et al., 2013). However, plants produce a vast range of different tannin types and concentrations (Mueller-Harvey, 2006) and the positive or negative effects of this compound appears to depend on the type and level of CT in the plants (Barry and McNabb, 1999; Min et al., 2003), the amount ingested and the animal species involved (Frutos et al., 2004; Mueller-Harvey, 2006).

Sainfoin (*Onobrychis viciifolia*; forage in temperate regions) is one of the forage plants possessing CT that is considerably variable even within a single plant species (Marais et al., 2000). A recent study that evaluated a large sainfoin germplasm collection obtained from around the world reported a substantial variation in content and chemical structure of CT among sainfoin accessions (Stringano et al., 2012). Screening of those plants for their CT content and chemical characteristics with regard to their effects on ruminal CH_4 emission would be important. In addition, evaluating the effects of structural variation of semi-purified CT extracts obtained from selected sainfoin accessions on in vitro CH_4 production would be important before being investigated under in vivo conditions.

In addition to the three areas of research described above, an in vitro experiment alongside an in vivo experiment was conducted to establish the relationship between in vitro and in vivo CH_4 production. It is hypothesized that in vitro CH_4 measurement is related to the in vivo CH_4 production if both in vitro and in vivo CH_4 measurements are performed simultaneously, using the same animals as donor for microbial inoculum when they are fed and adapted to the same dietary material used as a substrate for in vitro incubation.

AIM AND OBJECTIVES

The overall aim of this thesis was to determine the effects of feeding strategies that are feasible in practice and may readily be adopted by dairy farmers, to reduce enteric methane emission of lactating dairy cows. Specific objectives were, to:

1. Evaluate the effects of starch varying in rate of fermentation and level of inclusion in concentrates on methane production of lactating dairy cows.
2. Examine the relationship between in vitro and in vivo methane production measured simultaneously, using inocula obtained from rumen-fistulated dairy cows adapted to the dietary treatments in vivo and incubating samples obtained from the diets tested in vivo.
3. Investigate the effects of maize silage made from whole-plant maize harvested different stage of maturity on methane emission of lactating dairy cows.
4. Evaluate the effects of N fertilisation rate and maturity of grass at harvest for silage making on methane emission of lactating dairy cows.
5. Screen the diversity of sainfoin accessions and assessing the structural variation of semi-purified condensed tannins extracts on in vitro methane production and fermentation characteristics.

OUTLINE OF THE THESIS

Research work presented in this thesis was part of the Low Emission Feed (LEF) and 'HealthyHay' projects. Within the LEF project, research related to source and quantity of starch in the diet, grass quality (herbage and grass silage), feed additives and their interaction effects on enteric CH_4 production of dairy cows were investigated. Research in this thesis focuses mainly on the effects of source and level of starch in the diet, quality of grass silage, and condensed tannins (CT) on CH_4 production in dairy cows. In all in vivo experiments, CH_4 emission was measured using an open-circuit indirect calorimetry system as the 'golden standard' method to obtain accurate measurements of daily emissions of CH_4 from individual dairy cows. In addition, milk yield was measured to enable quantification of the effects of feeding strategies on CH_4 emission intensity. Feed digestibility, N and energy balance were measured during the period the cows were housed in the climate-controlled respiration chambers. In addition, in situ ruminal degradation characteristics of concentrates, grass and maize silages were determined using lactating dairy cows fitted with ruminal cannula.

This thesis comprises eight chapters of which six chapters are dedicated to research experiments conducted to evaluate the effects of different feeding strategies on enteric CH_4 emissions of dairy cows. The first experiment in **Chapter 2** involves investigation of the effects of starch varying in rate of fermentation and its level of inclusion in the concentrates on CH_4 production of dairy cows. In relation with the experiment described in Chapter 2, and to establish the relationship between in vitro and in vivo CH_4 production of dairy cows, the study reported in **Chapter 3** compared the in vitro and in vivo CH_4 measurements from different types and levels of starch in the diet when both CH_4 measurements were performed simultaneously, using the same cows as donor for microbial inoculum when they were fed and adapted to the same dietary material used as a substrate for in vitro incubation. Next to type and level of starch in the concentrates, the effects of quantity and quality of starch from roughage on CH_4 emission was investigated using whole-plant maize silage made from maize crops harvested at four sequential stages of maturity (**Chapter 4**). In a subsequent experiment (**Chapter 5**), the effect of N fertilisation rate and maturity of ryegrass at harvest on CH_4 emission of lactating dairy cows fed grass silage based diets was examined. And to screen the effect of variation in structural composition of CT in sainfoin accessions on CH_4 production, the in vitro gas production technique was used (**Chapter 6**). As a continuation of the study in Chapter 6, the anti-methanogenic effect of semi-purified CT extracts obtained from selected sainfoin accessions were examined and presented in **Chapter 7**. Based on the collective

findings from all experiments conducted in this thesis, the implication of feeding strategies as an opportunity to mitigate enteric CH_4 emissions from dairy cows is discussed in **Chapter 8**.

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CHAPTER 2

Effects of dietary starch content and rate of fermentation on methane production in lactating dairy cows

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ABSTRACT

The objective of this study was to investigate the effects of starch varying in rate of fermentation and level of inclusion in the diet in exchange for fibre on methane (CH_4) production of dairy cows. Forty Holstein-Friesian lactating dairy cows of which 16 were rumen cannulated grouped in 10 blocks of four cows each. Cows received diets consisting of 60% grass silage and 40% concentrate (dry matter basis). Cows within block were randomly assigned to one of four different diets composed of concentrates that varied in rate of starch fermentation (slowly (S) vs. rapidly (R) rumen fermentable; native vs. gelatinized maize grain) and level of starch (low vs. high; 270 vs. 530 g/kg of concentrate dry matter). Results of rumen in situ incubations confirmed that the fractional rate of degradation of starch was higher for R than S starch. Effective rumen degradability of organic matter (OM) was higher for high than low starch and higher for R than S starch. Increased level of starch, but not starch fermentability, decreased dry matter intake (DMI) and daily CH_4 production. Milk yield (mean 24.0 ± 1.02 kg/d), milk fat content (mean 5.05 ± 0.16%) and milk protein content (mean 3.64 ± 0.05%) did not differ between diets. Methane expressed per kilogram of fat- and protein-corrected milk, per kilogram of DMI or as a fraction of gross energy intake did not differ between diets. Methane expressed per kilogram of estimated rumen-fermentable OM (eRFOM) was higher for S than R starch based diets (47.4 vs. 42.6 g/kg of eRFOM) and for low than high starch-based diets (46.9 vs. 43.1 g/kg of eRFOM). Apparent total-tract digestibility of neutral detergent fibre and crude protein were not affected by diets, but starch digestibility was higher for diets based on R starch (97.2%) compared with S starch (95.5%). Both total volatile fatty acid concentration (109.2 vs. 97.5 mM) and propionate proportion (16.5 vs. 15.8 mol/100 mol) was higher for R starch- compared with S starch-based diets, but unaffected by the level of starch. Total N excretion in feces plus urine and N retained were unaffected by dietary treatments, and similarly energy intake and output of energy in milk expressed per unit metabolic body weight were not affected by treatments. In conclusion, an increased rate of starch fermentation and increased level of starch in the diet of dairy cattle reduced CH_4 produced per unit of eRFOM but did not affect CH_4 production per unit of feed DMI or per unit of milk produced.

Key words: methane; starch fermentability; starch level; dairy cow

INTRODUCTION

Starch is a major source of glucogenic energy for high-yielding dairy cows and a source of fermentable energy for rumen microorganisms (Koenig et al., 2003). In addition to carbon dioxide, microbial matter and VFA production, fermentation of feeds in the rumen results in release of hydrogen which is utilized by methanogenic archaea to reduce carbon dioxide and produce methane (CH_4), a potent greenhouse gas that has 25 times more global warming potential than carbon dioxide (IPCC, 2007). The production of CH_4 is influenced by dietary factors, such as type and amount of feed, and various dietary strategies have been suggested to reduce enteric CH_4 production (Hristov et al., 2013; Knapp et al., 2014). Compared with dietary fibre, starch fermentation in the rumen may result in reduced enteric CH_4 production because fermentation of starch favours production of propionate (Bannink et al., 2006), creating an alternative hydrogen sink to methanogenesis. Moreover, unlike fibre and sugar, a substantial fraction of potentially fermentable starch may escape from rumen fermentation to be digested enzymatically in the small intestine adding to the energy supply of the animal without associated losses of energy with CH_4 production (Dijkstra et al., 2011).

The use of starch versus fibre, as well as increasing dietary starch content in the concentrate are potential options to reduce ruminal CH_4 production, relative to total energy supply to the animal (Hristov et al., 2013). Using a modelling approach, Benchaar et al. (2001) estimated that the use of less ruminally fermentable starch and replacing fibrous concentrate with starchy concentrate reduces CH_4 emissions by 17 and 22% in ruminants, respectively. Compared with rapidly fermentable starch sources such as barley or wheat, the use of a slowly fermentable starch such as maize may result in a reduction of CH_4 production (Mills et al., 1999, 2001), mainly attributed to a shift in starch digestion from the rumen to the small intestine. Similarly, the substitution of sugar in the concentrate with a rapidly fermentable starch source such as barley or wheat was estimated to reduce CH_4 production in ruminants (Mills et al., 2001), due to lower ratio of acetate and butyrate to propionate production from starch fermentation (Benchaar et al., 2001, Mills et al., 2001) and the subsequent increase in ME supply to the dairy cow (Mills et al., 2001). However, increasing the amount of rapidly fermentable starch in the diet at the expense of forage fibre can increase the production of VFA beyond the buffering and absorptive capacity of the rumen, leading to a decreased ruminal pH that has negative consequences on fibre degradation and production of dairy cows (Dijkstra et al., 2012). Moreover, the level of abatement of enteric CH_4

production achievable in dairy cattle with grass silage-based diets in which sources and levels of starch in the concentrate vary is largely lacking.

The objective of this study was to evaluate the effects of starch varying in rate of fermentation and in level of inclusion in concentrate that accounted for 40% of the total mixed rations DM on CH_4 production of dairy cows. We hypothesized that increasing the inclusion of ruminally fermentable starch in the diet at the expense of fibre enhances propionate production, and that decreasing rate of fermentation of the starch in the rumen shifts starch digestion from the rumen to the small intestine, both expected to decrease CH_4 production expressed per unit of feed or milk.

MATERIALS AND METHODS

This experiment was conducted as a complete randomized block design at the animal research facility of Wageningen University (Wageningen, The Netherlands). All experimental procedures were approved by the Institutional Animal Care and Use Committee of Wageningen University and carried out under the Dutch Law on Animal Experimentation.

Cows, experimental design and diets

Forty multiparous lactating Holstein-Friesian dairy cows were selected and grouped in 10 blocks based on parity (2.9 ± 1.1; mean ± SD), DIM (215 ± 89 d), fat- and protein-corrected milk (FPCM; 35.9 ± 9.5 kg/d) at the start of the experiment, and presence or absence of rumen cannula. Sixteen cows were rumen cannulated and used to evaluate the effects of dietary treatments on rumen fermentation characteristics (pH and VFA concentration). Cows within a block were randomly assigned to one of four dietary treatments. Treatments were 1) 270 g of slowly fermentable starch per kilogram of concentrate DM; 2) 530 g of slowly fermentable starch per kilogram of concentrate DM; 3) 270 g of rapidly fermentable starch per kilogram of concentrate DM; and 4) 530 g of rapidly fermentable starch per kilogram of concentrate DM. Cows were fed a total mixed diet composed of grass silage and concentrate mixed at 60:40 ratios (DM basis). Diets were offered individually and in equal portions during a.m. and p.m. (0600 and 1600 h) feedings. The concentrates were in meal form and mixed with the forage portion manually when fed.

The primary starch sources in the concentrate were native maize grain, which is slowly fermentable (S), and gelatinized maize grain, which is rapidly fermentable (R), and each source was included at two levels: a low (L; 270 g of starch per kilogram of concentrate DM) and a high (H; 530 g starch per kilogram of concentrate DM) level. Increasing the level of

starch in concentrate was achieved by exchanging either ground native maize grain or ground gelatinized maize grain with beet pulp and palm kernel expeller on DM basis. The ingredient composition of the concentrates is shown in Table 1. Both native and gelatinized maize grains were obtained from a single batch of maize. These two starch sources were chosen to create a large difference in rate of starch fermentation.

The experiment was conducted in 10 successive periods of 17 d each. In each period, cows were individually housed in tie-stalls for 12 d as an adaptation period and to determine individual daily feed intake. Diets were supplied ad libitum for the first 8 d in the tie-stalls (approximately at 110% of expected voluntary intake). From d 9 to 17, feed intake was restricted per block to 95% of the ad libitum feed intake of the animal consuming the lowest amount of feed during d 3 to 8 within a block to avoid the potential confounding effect of feed intake level on CH_4 measurements. After the end of the adaptation period, cows were housed for 5 d in one of the two identical climate-controlled respiration chambers for the measurement of CH_4 production. In addition, digestibility measurements and a complete N and energy balance were performed. Because two chambers were available, measurements were obtained in 10 periods, staggered in time in an incomplete randomized block design, as described previously by Van Zijderveld et al. (2011b). Within each period, two cows receiving the same treatment were housed in one chamber, and two cows receiving a different treatment were housed in the other chamber. Within each chamber, the two cows originated from different blocks and each dietary treatment was not paired with the other dietary treatments in equal number of periods. The experimental unit for data measured in the respiration chambers (in particular gaseous exchange, N and energy balance parameters) therefore consisted of a pair of cows. The respiration chambers have been described in detail by Verstegen et al. (1987) and Van Zijderveld et al. (2011a). Cows had free access to drinking water throughout the experiment.

Ruminal pH and concentration of VFA

On d 10 and 11 of each experimental period, equal volumes of rumen fluid from rumen-cannulated cows were collected to determine rumen pH and VFA concentration. Rumen fluid for each cow was collected from the front ventral, middle ventral and cranial dorsal sac of the rumen (Abrahamse et al., 2008) at 0 (just before the 0600 h a.m. feeding), 1, 2, 3, 4, 6, and 8 h after a.m. feeding. Rumen fluid was collected by suction method using a solid plastic tube (0.85 m long and 2.5 cm in diameter) perforated at the end. Ruminal pH was determined immediately after sampling using a portable pH meter (Hanna Instruments Model HI 9024,

Table 1. Ingredients and nutrient composition (g/kg of DM, unless otherwise stated) of concentrates fed during the experiment.

	Slowly fermentable		Rapidly fermentable	
Item	Low level	High level	Low level	High level
Ingredient				
Native maize grain	412.9	816.1	–	–
Gelatinized maize grain	–	–	412.9	816.1
Palm kernel expeller	266.8	119.8	266.8	119.8
Beet pulp (low sugar)	282.7	–	282.7	–
Soybean meal	18.7	–	18.7	–
Soybean meal, formaldehyde treated	–	13.5	–	13.5
Urea	13.5	23.4	13.5	23.4
Limestone	0.9	14.0	0.9	14.0
Magnesium oxide	–	5.0	–	5.0
Salt	3.1	3.7	3.1	3.7
Sodium bicarbonate	–	2.5	–	2.5
Vitamin-mineral premix[1]	1.5	1.5	1.5	1.5
Nutrient composition				
DM (g/kg product as fed)	884	889	887	882
Ash	54	50	47	48
CP	169	170	167	186
NDF	311	170	308	153
ADF	187	90	181	80
ADL	41	21	41	23
Crude fat	43	34	47	41
Starch	275	518	303	542
Sugar	36	22	33	22
Gross energy (MJ/kg of DM)	18.5	18.4	18.7	18.3

[1]Contained per kilogram of premix: 4,000,000 IU of vitamin A; 833,000 IU of vitamin D; 10,000 mg of vitamin E; 10,000 mg of Cu; 23,333 mg of Zn; 18,467 mg of Mn; 500 mg of Co; 667 mg of I, and 200 mg of Se.

IJsselstein, The Netherlands). A subsample (0.75 ml) of ruminal fluid was mixed with an equal volume of meta-phosphoric acid and immediately stored at −20°C pending VFA analysis.

Feed intake, nutrients digestibility, N and energy balance

Samples of grass silage and concentrates were collected when feeds were prepared. During the CH_4 measurement period, orts (when present) were weighed daily and stored at 4°C. At the end of each period, daily orts were composited per cow, mixed and a subsample was retained and stored at −20°C. Apparent total-tract digestibility of nutrients was determined using chromium oxide (Cr_2O_3) as an external marker included in the concentrate. The marker (1.5 g Cr_2O_3/kg of concentrate DM) was supplied for each cow starting on day one of the experimental period. During the CH_4 measurement period, grab samples of feces (ca. 300 g) were collected daily during milking and stored at −20°C. Prior to freeze drying, the samples were pooled per cow, mixed and a subsample was taken.

The body weight of cows was taken on the first (d 13) and the last day (d 17) of the measurement period. The animals in the chamber were tethered in individual stalls complete with slatted floor fitted for collection of the manure (mixture of feces and urine). For complete N balance determination, the manure produced by the two cows in a chamber during the 5-d period was quantitatively collected, weighed, mixed thoroughly, subsampled and stored at −20°C pending analysis. Also, N volatilized in the form of ammonia that may result from mixing of feces and urine was obtained from samples of condensed water (i.e. collected from the heat exchanger) and 25% sulphuric acid solution wt/wt (i.e. through which the outflowing air was led to trap aerial ammonia) of each chamber.

Milk yield and milk composition

Cows were milked twice daily (0600 and 1600 h), and milk yield was recorded during the 5 d of CH_4 measurement period. During each milking samples were collected in duplicate. Morning and afternoon samples were collected separately into tubes containing sodium azide and stored no longer than 4 d at +4°C pending fat-, protein- and lactose-contents analysis, and determination of SCC concentration. The second sample was pooled per cow based on a weight basis proportional to milk yield (5 g/kg of milk) and stored at −20°C until analysed for urea, energy and N content.

In situ rumen degradation of diets

Ruminal degradability of starch, OM and N in concentrate was determined in a separate experiment using three rumen cannulated lactating Holstein-Friesian dairy cows. The cows were 387.0 ± 7.8 DIM and producing 22.8 ± 3.9 kg/d of milk. Cows were fed ad libitum a mixed ration of 50% grass silage (CP, 104 g/kg of DM; NDF, 516 g/kg of DM) and 50%

maize silage (CP, 72 g/kg of DM; NDF, 397 g/kg of DM, starch, 374 g/kg of DM) and a commercial concentrate (160 g/kg of DM starch, 200 g/kg of DM CP, 38 g/kg of DM crude fat, and 80 g/kg of DM ash) according to milk production up to a maximum of 8 kg/d. Only concentrate samples were incubated in the rumen. The effective rumen degradability (ERD) of OM in the grass silage was estimated by near infrared spectroscopy analysis (BLGG AgroXpertus, Wageningen, The Netherlands). Nylon bags were prepared according to the Dutch in situ protocol (Tas et al., 2006). Briefly, nylon bags with an inner size of 10 x 8 cm, a pore size of 40 μm and porosity of 0.30 (PA 40/30, Nybolt, Switzerland) were filled with approximately 5 g (DM basis) concentrate ground to pass a 3-mm sieve. Three bags per incubation time for each concentrate were incubated for 2, 4, 6, 8, 12, 24, and 48 h in the rumen of each cow using the all-in all-out procedure. After incubation, bags were immediately placed in ice water for approximately 5 min to stop fermentation, and rinsed with tap water. The bags were then washed in a washing machine (AEG Turnamat SL, Nuremberg, Germany) for 40 min in cold water (gentle wool-wash programme without centrifuging) and freeze-dried. Dried samples were weighed, pooled to one sample per incubation time per cow, ground through a 1-mm sieve (Peppink 100AN, Olst, The Netherlands) and analysed for DM, ash, N, and starch.

To determine the rumen degradation characteristics of OM, N and starch in the concentrates, residues per incubation time per cow were fitted to a first-order nonlinear model: $Y(t) = U + D \times e^{-kd \times t}$; where Y(t) = proportion of total residue present at time t (g/kg); U = the truly undegradable fraction (g/kg) (for OM and N only); D = the potentially degradable fraction (g/kg); t = time of incubation (h); and k_d = the fractional rate of degradation of the D fraction (/h). The NLIN procedure of SAS (SAS Institute Inc., 2010) was used to estimate the parameter values, with D, U and k_d constrained to be positive. The ERD of starch, OM and N in the concentrates was calculated as described by Ørskov and McDonald (1979) using the formula: $ERD = W + (D \times k_d)/(k_d + k_p)$ and assuming a fractional ruminal outflow rate (k_p) of 0.06 /h for concentrates (Tamminga et al., 1994). The washout (W) fraction (g/kg) which is assumed to be rapidly degradable was calculated as 1,000 − D − U. The estimated rumen fermentable OM (eRFOM) in the total mixed diets was calculated using the ERD of OM of concentrates obtained from the rumen incubations and the estimated ERD of OM of grass silage, by taking into account the OM content of grass silage and each concentrate, and the proportion of these diet ingredients.

Analytical procedures

All samples of feeds, orts, feces and manure were freeze dried and ground to pass through a 1-mm sieve using a Wiley mill (Peppink 100AN) before analysis, except for N analysis in manure and NH_3-N analysis in the silage for which fresh samples were used. Feed and feces samples were analysed for DM (ISO 6496, 1999), ash (ISO 5984, 2002), N (ISO 5983, 2005), crude fat (ISO 6492, 1999), starch (ISO 15914, 2004) and gross energy (GE; ISO 9831, 1998). Crude protein content was calculated as N × 6.25. NDF was analysed according to Van Soest et al. (1991) after pre-treatment with amylase. Acid detergent fibre and ADL were determined according to Van Soest (1973). Determination of sugar content in the feed was based on the method described by Van Vuuren et al. (1993). Concentrate and feces samples were analysed for chromium using atomic absorption spectrophotometry (Williams et al., 1962). Milk protein, fat and lactose contents, and SCC concentration were determined according to ISO 9622 (1999) at VVB (VVB, Doetinchem, The Netherlands), and milk urea was determined using the pH difference technique (ISO 14637, 2004).

For determination of the concentration of VFA, the frozen ruminal fluid samples were thawed and centrifuged at 10,000 × g for 10 min at 4^{o}C. The supernatant was transferred to a gas-chromatography vial for analysis of VFA concentration using gas chromatography (Fisons HRGC MEGA2, CI instruments, Milan, Italy) according to the method described by Taweel et al. (2005).

Statistical analysis

In general, statistical analysis were carried out by ANOVA using the PROC MIXED procedure in SAS (SAS Institute Inc., 2010) using various models. The data for two cows fed on the diet composed of 270 g of rapidly fermentable starch per kilogram of concentrate DM in one respiration chamber had to be excluded from all analysis due to malfunctioning of chamber and unreliable data. Daily data were averaged per experimental unit per period before statistical analysis. Cow was considered as the experimental unit for all measurements except for CH_4 production parameters, N and energy balance traits for which a pair of cows housed in the same respiration chamber was considered to be the experimental unit. For CH_4-production parameters, and N and energy balance traits, the model included the fixed effects of respiration chamber, starch source, level of starch, and source × level interaction, and a random effect of period. Block was not included in the model, because the two cows housed within the same chamber originated from different blocks. For DMI, faecal digestibility, and milk characteristics the model included respiration chamber, block, starch source, level of

starch, and source × level interaction as fixed effects, and period as a random factor. For all analysis, the fixed effect of respiration chamber was initially included in the model but was removed from the model because it was not significant. Because of unequal variances, the Kenward−Roger option was used to estimate the denominator degrees of freedom. Autoregressive 1, compound symmetry and unstructured covariance structures were tested for each analysis. Depending on the characteristics of analysis, the covariance structure with the lowest Akaike's information criterion was selected (Littell et al., 1998), which in most cases was the compound symmetry covariance structure.

Similarly, data for ruminal pH and VFA concentration were analysed using the PROC MIXED procedure in SAS with block, starch source, level of starch, time, source × level, source × time, and level × time interactions as fixed effects and cow as a random effect using the repeated measures procedure with time as repeated measures. The relationship between measurements was assumed linear. The covariance structure was defined in the model as being spatial power for unequally spaced measurement times.

Data on in situ rumen degradation of concentrates and diets were similarly analysed using the PROC MIXED procedure in SAS with starch source, level of starch, and source × level interaction as fixed effects, and cow as random effect.

All results are reported as least square means. Significance of effect was declared at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$.

RESULTS

Chemical composition of concentrates and diets

The chemical composition of concentrates, grass silage and total mixed diets is presented in Table 1 and Table 2. The nutrient composition in the concentrates had slight differences, in particular in starch content. Starch content in concentrates with rapidly fermentable (R) starch had a 26 g/kg of DM higher starch content than concentrates with slowly fermentable (S) starch, resulting in an average 11 g/kg of DM higher starch content in total mixed diets containing R starch compared with S starch. The analysed GE content of grass silage was somewhat higher than expected and higher than that of concentrates. Since the proportion of grass silage in the total diet did not differ between treatments, the high GE content of the silage does not affect differences between treatments.

Table 2. Analysed chemical composition of grass silage and calculated chemical composition of total mixed diets (g/kg of DM, unless otherwise stated).

		Total mixed diets[1]			
Item	Grass silage	SL	SH	RL	RH
DM (g/kg of product as fed)	512	664	666	663	659
Ash	88	74	72	71	72
CP	148	156	157	156	163
NDF	528	441	385	440	378
ADF	296	252	214	250	210
ADL	20	29	21	29	21
Crude fat	38	40	36	42	39
Starch	NA[2]	110	207	121	217
Sugar	79	62	56	61	56
Gross energy (MJ/kg of DM)	19.1	18.8	18.8	18.9	18.7

[1]Calculated based on analysed chemical composition of grass silage and concentrate and mixed at 60:40 ratio (on DM basis). SL, diet containing 270 g of slowly fermentable starch per kilogram of concentrate DM; SH, diet containing 530 g of slowly fermentable starch per kilogram of concentrate DM; RL, diet containing 270 g of rapidly fermentable starch per kilogram of concentrate DM; RH, diet containing 530 g of rapidly fermentable starch per kilogram of concentrate DM.
[2]NA, starch was not analysed in the grass silage.

In situ rumen degradation characteristics

Starch source affected all degradation characteristics of starch, N and OM (Table 3). Concentrates composed of R starch had about three times higher ($P < 0.001$) k_d of starch than concentrates based on S starch (average = 0.155 /h vs. 0.054 /h, respectively) and a much higher W fraction (average = 315 vs. 64 g/kg, respectively; $P < 0.001$), leading to a 59% higher ERD of starch. A significant interaction between source and level of starch indicated that the effect of source of starch on W, D, k_d and ERD of starch was more pronounced with low starch levels than with high starch levels in the concentrate. The effects of starch resulted in a lower ERD of OM and N for S starch- compared with R starch-based concentrates and a higher ERD with H compared with L. These effects were less pronounced with L compared with H for ERD of starch. The calculated eRFOM in a total mixed diet was lower for S starch than for R starch, and lower for L starch than for H starch ($P < 0.001$), but a significant interaction between source and level of starch indicated that the effects are not additive.

Table 3. In situ rumen degradation characteristics of the concentrates used in the experiment and estimated effective rumen degradability of OM in the total mixed diet.

	Concentrate[1]					*P*-value		
Item	SL	SH	RL	RH	SED[2]	Source (S)	Level (L)	S × L
Concentrate								
Starch								
W[3]	38	89	343	286	21.2	<0.001	0.861	0.011
D[4]	963	911	657	714	21.2	<0.001	0.861	0.011
k_d[5]	0.054	0.054	0.173	0.137	0.0104	<0.001	0.037	0.039
ERD[6]	495	521	830	782	12.1	<0.001	0.238	0.005
Organic matter								
W	116	167	187	224	8.7	<0.001	<0.001	0.280
D	831	818	667	622	24.0	<0.001	0.139	0.375
U[7]	54	15	146	154	21.7	<0.001	0.351	0.175
k_d	0.043	0.041	0.078	0.139	0.0066	<0.001	0.001	0.001
ERD	459	500	564	660	9.4	<0.001	<0.001	0.005
Nitrogen								
W	299	471	363	536	12.3	0.001	<0.001	0.938
D	701	529	637	464	12.3	0.001	<0.001	0.938
k_d	0.025	0.024	0.025	0.028	0.0039	0.033	0.197	0.049
ERD	504	623	552	681	4.2	<0.001	<0.001	0.135
Total mixed diet								
eRFOM[8]	494	510	536	575	3.9	<0.001	<0.001	<0.001

[1]SL, concentrate containing 270 g of slowly fermentable starch per kilogram of concentrate DM; SH, concentrate containing 530 g of slowly fermentable starch per kilogram of concentrate DM; RL, concentrate containing 270 g of rapidly fermentable starch per kilogram of concentrate DM; RH, concentrate containing 530 g of rapidly fermentable starch per kilogram of concentrate DM.
[2]SED, standard error of the difference of means.
[3]W, washable fraction (g/kg of respective nutrient).
[4]D, potentially degradable fraction (g/kg of respective nutrient).
[5]k_d, fractional degradation rate (per h) of potential degradable fraction.
[6]ERD, effective rumen degradability (g/kg of respective nutrient).
[7]U, undegradable fraction (g/kg of respective nutrient) and estimated to be zero for starch and N.
[8]eRFOM, estimated rumen-fermentable OM in the total mixed diets (g/kg of OM) was calculated using the ERD of OM of concentrates obtained from the rumen incubations and the estimated ERD of OM in the grass silage estimated by near-infrared spectroscopy analysis.

Ruminal pH and concentration of VFA

The effects of source and level of starch in the diet on rumen pH, VFA concentration and VFA molar proportions are presented in Table 4. Mean ruminal pH was not affected by either starch rate of fermentation or by level of starch in the diets, whereas total VFA concentration was higher for diets based on R starch than on S starch (109.2 vs. 97.5 mM, $P = 0.002$). Both source and level of starch in the diet did not affect VFA molar proportions, except for higher propionate proportions ($P = 0.046$), and a trend for lower isobutyrate proportions ($P = 0.051$) and acetate:propionate ratio ($P = 0.054$) with the R starch- compared with the S starch-based diets. On average for all dietary treatments, the pH decreased from the prefeeding value of 6.73 to 6.41 and 6.33 at 3 and 4 h after a.m. feeding, respectively (Figure 1). Rumen total VFA concentrations (Figure 2) and acetate:propionate ratio (Figure 3) varied with time of sampling relative to a.m. feeding. The interactions between time and source or level of starch were not significant, except for rumen pH ($P < 0.001$).

Table 4. Rumen fluid pH and concentration of VFA of lactating dairy cows fed diets that differed in starch rate of fermentation and level of inclusion in concentrate.

	Diet[1]					*P*-value		
Item	SL	SH	RL	RH	SED[2]	Source (S)	Level (L)	S × L
Rumen pH	6.53	6.49	6.51	6.53	0.088	0.872	0.889	0.650
Total VFA (mM)	101.1	93.9	110.9	107.4	4.39	0.002	0.107	0.555
VFA (mol/100 mol)								
Acetate (A)	68.3	69.1	68.6	67.9	0.55	0.272	0.859	0.069
Propionate (P)	15.9	15.6	16.2	16.8	0.46	0.046	0.684	0.172
Butyrate	11.5	11.2	11.1	11.8	0.32	0.791	0.358	0.052
Isobutyrate	1.12	0.88	0.80	0.66	0.19	0.051	0.164	0.691
Valerate	1.54	1.54	1.54	1.63	0.11	0.550	0.580	0.520
Isovalerate	1.69	1.64	1.82	1.29	0.31	0.637	0.215	0.281
A:P	4.31	4.45	4.27	4.06	0.15	0.054	0.725	0.107

[1]n = 4 for all diets except for RL for which n = 3. SL, diet containing 270 g of slowly fermentable starch per kilogram of concentrate DM; SH, diet containing 530 g of slowly fermentable starch per kilogram of concentrate DM; RL, diet containing 270 g of rapidly fermentable starch per kilogram of concentrate DM; RH, diet containing 530 g of rapidly fermentable starch per kilogram of concentrate DM.

[2]SED, standard error of the difference of means.

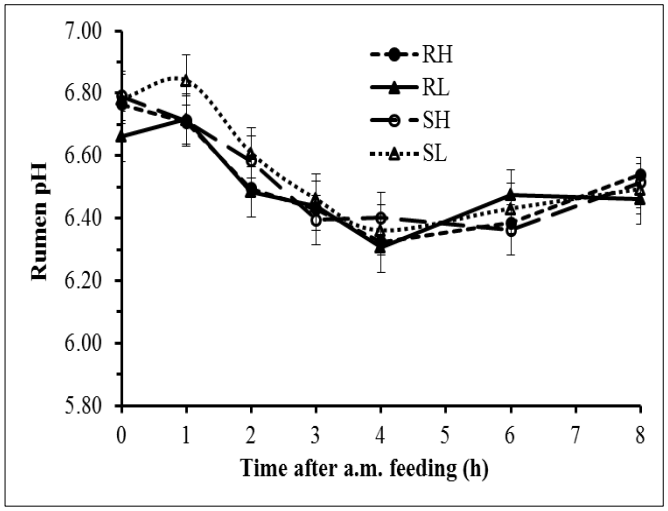

Figure 1. Effects of source and level of starch in the diet of lactating dairy cows on rumen pH as a function of time after a.m. feeding. SL, diet containing 270 g of slowly fermentable starch per kilogram of concentrate DM; SH, diet containing 530 g of slowly fermentable starch per kilogram of concentrate DM; RL, diet containing 270 g of rapidly fermentable starch per kilogram of concentrate DM; RH, diet containing 530 g of rapidly fermentable starch per kilogram of concentrate DM. Effect of time ($P < 0.001$), and interactions of source of starch with time ($P = 0.143$) and level of starch with time ($P = 0.027$).

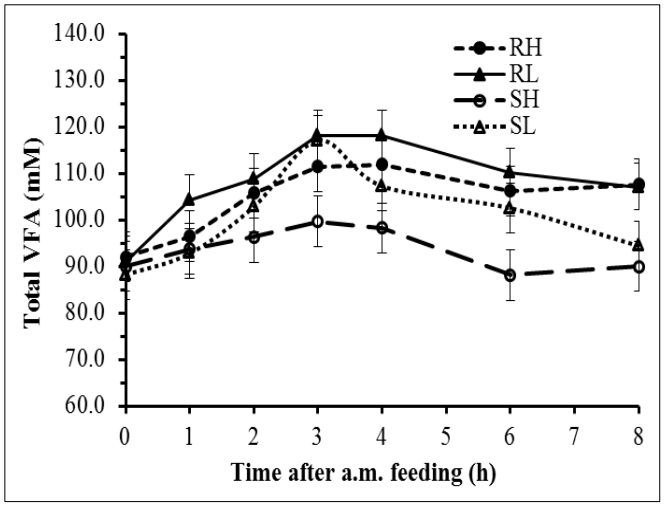

Figure 2. Effects of source and level of starch in the diet of lactating dairy cows on rumen total VFA concentration as a function of time after a.m. feeding. SL, diet containing 270 g of slowly fermentable starch per kilogram of concentrate DM; SH, diet containing 530 g of slowly fermentable starch per kilogram of concentrate DM; RL, diet containing 270 g of rapidly fermentable starch per kilogram of concentrate DM; RH, diet containing 530 g of rapidly fermentable starch per kilogram of concentrate DM. Effect of time ($P < 0.001$), and interactions of source of starch with time ($P = 0.725$) and level of starch with time ($P = 0.592$).

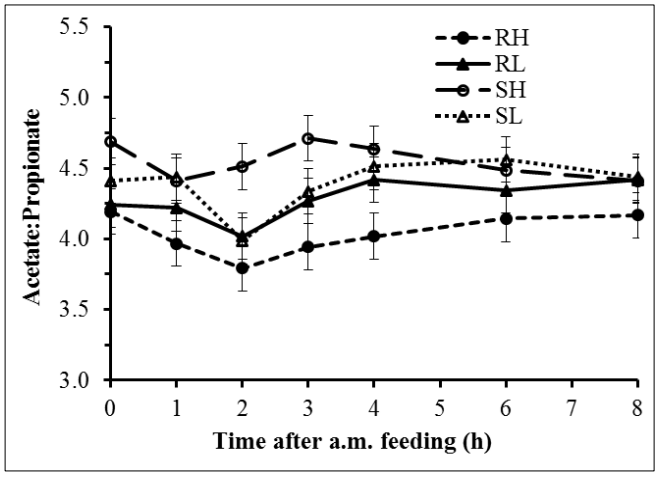

Figure 3. Effects of source and level of starch in the diet of lactating dairy cows on rumen acetate:propionate ratio as a function of time after a.m. feeding. SL, diet containing 270 g of slowly fermentable starch per kilogram of concentrate DM; SH, diet containing 530 g of slowly fermentable starch per kilogram of concentrate DM; RL, diet containing 270 g of rapidly fermentable starch per kilogram of concentrate DM; RH, diet containing 530 g of rapidly fermentable starch per kilogram of concentrate DM. Effect of time ($P = 0.002$), and interactions of source of starch with time ($P = 0.928$) and level of starch with time ($P = 0.147$).

For all figures, error bars represent the standard error of the difference of means.

Feed intake and nutrient digestibility

Dry matter intake and apparent total-tract nutrient digestibility are presented in Table 5. Dry matter intake was 0.8 kg/d lower with the high level of starch (P = 0.022), but was unaffected by source of starch in the diet. Dry matter intake in the chambers (mean = 19.0 kg/d) did not differ from DMI during the last 3 days of the adaptation period in the tie-stalls (mean = 18.9 kg/d). Apparent total-tract digestibility of NDF and CP was not affected by dietary treatments. However, for both DM and OM digestibility as well as for crude fat and GE digestibility, significant interactions existed between source and level of starch in the diet, showing a higher digestibility with increased starch level of R starch but a lower digestibility with increased starch level for S starch. Diets based on R starch had a 1.7% higher starch digestibility compared with S starch-based diets (P = 0.006). The level of starch in the diet had no effect on starch digestibility.

Table 5. Dry matter intake and apparent total-tract digestibility of nutrients in lactating dairy cows fed diets that differed in starch rate of fermentation and level of inclusion in concentrate.

	Diet[1]					*P*-value		
Item	SL	SH	RL	RH	SED[2]	Source (S)	Level (L)	S × L
DMI (kg/d)	19.4	18.5	19.4	18.6	0.51	0.970	0.022	0.791
Digestibility (%)								
DM	72.9	69.7	67.2	72.7	1.82	0.302	0.377	0.002
OM	74.9	71.1	68.9	74.4	1.81	0.299	0.509	0.001
CP	63.0	63.0	59.0	64.3	2.02	0.348	0.074	0.074
NDF	72.8	71.0	69.9	69.5	2.05	0.140	0.456	0.631
Crude fat	63.9	56.4	61.3	64.7	2.21	0.081	0.203	0.002
Starch	95.6	95.4	96.7	97.6	0.01	0.006	0.491	0.237
Gross energy	69.5	67.5	65.6	69.8	1.75	0.523	0.381	0.019

[1]n = 10 for SL, SH and RH, and n = 8 for RL. SL, diet containing 270 g of slowly fermentable starch per kilogram of concentrate DM; SH, diet containing 530 g of slowly fermentable starch per kilogram of concentrate DM; RL, diet containing 270 g of rapidly fermentable starch per kilogram of concentrate DM; RH, diet containing 530 g of rapidly fermentable starch per kilogram of concentrate DM.
[2]SED, standard error of the difference of means.

Milk yield and milk composition

Daily milk yield (average 24.0 kg/d), and content of fat (5.05%), protein (3.64%), lactose (4.53%) and milk urea (3.51 mmol/l) were not influenced by either source or level of starch in

diet (Table 6). Similarly, SCC concentration was not affected by either source or level of starch in the diet. The FPCM and milk fat yield showed a tendency (P = 0.092 and P = 0.077, respectively) to be higher for diets containing a low level of starch. Milk protein yield showed a tendency (P = 0.088) to be higher for cows fed diets based on R starch compared with S starch.

Table 6. Milk yield and milk composition of dairy cows fed diets that differed in starch rate of fermentation and level of inclusion in concentrate.

	Diet[1]					*P*-value		
Item	SL	SH	RL	RH	SED[2]	Source (S)	Level (L)	S × L
Yield (kg/d)								
Milk	24.5	22.5	24.4	24.7	1.34	0.276	0.390	0.238
FPCM[3]	27.9	25.2	27.1	26.9	1.17	0.614	0.092	0.146
Fat	1.25	1.11	1.17	1.15	0.061	0.643	0.077	0.223
Protein	0.87	0.79	0.88	0.88	0.039	0.088	0.155	0.202
Lactose	1.11	1.03	1.09	1.13	0.063	0.393	0.710	0.203
Composition								
Fat (%)	5.24	5.11	4.97	4.88	0.313	0.271	0.602	0.930
Protein (%)	3.61	3.58	3.68	3.68	0.154	0.428	0.870	0.866
Lactose (%)	4.53	4.55	4.47	4.55	0.065	0.486	0.236	0.486
Urea (mmol/l)	3.60	3.49	3.46	3.50	0.185	0.619	0.809	0.587
SCC × 1,000 (cells/ml)	203	165	237	195	84.7	0.597	0.519	0.975

[1]n = 10 for SL, SH and RH, and n = 8 for RL. SL, diet containing 270 g of slowly fermentable starch per kilogram of concentrate DM; SH, diet containing 530 g of slowly fermentable starch per kilogram of concentrate DM; RL, diet containing 270 g of rapidly fermentable starch per kilogram of concentrate DM; RH, diet containing 530 g of rapidly fermentable starch per kilogram of concentrate DM.

[2]SED, standard error of the difference of means.

[3] Fat- and protein-corrected milk = (0.337 + 0.116 × fat (%) + 0.06 × protein (%)) × milk yield (kg/d).

Dietary treatments and methane production

Daily CH_4 production was not affected by source of starch but was higher at low starch levels than at a high starch levels (432 vs. 399 g/d, respectively; P = 0.017; Table 7). Methane production per kilogram of milk, per kilogram of FPCM, per kilogram of DMI, per kilogram of digested DM and as percentage of GE intake was not influenced by dietary treatments. Ho-

Table 7. Methane production of lactating dairy cows fed diets that differed in starch rate of fermentation and level of inclusion in concentrate.

	Diet[1]					*P*-value		
Item	SL	SH	RL	RH	SED[2]	Source (S)	Level (L)	S × L
CH_4 (g/d)	436	397	427	401	16.3	0.862	0.017	0.606
CH_4 (g/kg of DMI)	22.4	21.5	22.2	21.6	0.65	0.852	0.131	0.732
CH_4 (g/kg of milk)	17.8	17.9	17.8	16.6	0.95	0.346	0.389	0.345
CH_4 (g/kg of FPCM[3])	15.7	15.9	15.9	15.0	0.59	0.450	0.476	0.196
CH_4 (g/kg of digested DM)	30.5	30.7	33.0	29.9	1.24	0.339	0.119	0.090
CH_4 (g/kg of eRFOM[4])	49.1	45.6	44.6	40.5	1.31	<0.001	0.002	0.770
CH_4 (% of GE intake)	6.6	6.3	6.5	6.4	0.20	0.898	0.112	0.587

[1]n = 5 for SL, SH and RH, and n = 4 for RL. SL, diet containing 270 g of slowly fermentable starch per kilogram of concentrate DM; SH, diet containing 530 g of slowly fermentable starch per kilogram of concentrate DM; RL, diet containing 270 g of rapidly fermentable starch per kilogram of concentrate DM; RH, diet containing 530 g of rapidly fermentable starch per kilogram of concentrate DM.
[2]SED, standard error of the difference of means.
[3]FPCM, fat- and protein-corrected milk.
[4]eRFOM, estimated rumen-fermentable organic matter.

wever, CH_4 expressed per kilogram of eRFOM was higher for diets based on S starch than R starch (47.4 vs. 42.6 g/kg of eRFOM, respectively; $P < 0.001$), and for diets based on L than H (46.9 vs. 43.1 g/kg of eRFOM, respectively; $P = 0.002$).

Nitrogen and energy balance

Nitrogen and energy balance data are presented in Table 8. Daily N intake was not affected by source and level of starch. Total N excretion in manure (feces and urine mixture) tended ($P = 0.064$) to be higher with diets based on R starch as compared with S starch. Diets based on R starch showed a tendency ($P = 0.060$) of higher secretion of N in milk as compared with diets based on S starch. There was a tendency ($P = 0.088$) of a lower secretion of N in milk for H compared with L in the diet. Nitrogen retention was not affected by level or source of starch in the diet. In addition, no significant differences were observed for GE intake, ME intake, heat production, milk energy output and total energy retention (all expressed per unit metabolic BW) between treatments.

Table 8. Nitrogen and energy balance in lactating dairy cows fed diets that differed in starch rate of fermentation and level of inclusion in concentrate.

	Diet[1]					*P*-value		
Item	SL	SH	RL	RH	SED[2]	Source (S)	Level (L)	S × L
Metabolic BW (kg of $BW^{0.75}$)	126.4	124.6	129.2	123.9	4.15	0.739	0.247	0.554
Gross energy intake (kJ/kg of $BW^{0.75}$ per day)	2,908	2,824	2,817	2,841	126.3	0.687	0.739	0.559
ME intake[3] (kJ/kg of $BW^{0.75}$ per day)	1,740	1,656	1,630	1,653	79.7	0.333	0.596	0.360
Methane production (kJ/kg of $BW^{0.75}$ per day)	192	178	184	181	7.9	0.670	0.140	0.360
Heat production (kJ/kg of $BW^{0.75}$ per day)	969	927	961	950	23.1	0.645	0.133	0.366
N intake (g/d)	489.1	474.6	479.6	484.0	14.93	0.993	0.643	0.389
N in manure (g/d)	322.7	325.2	341.7	352.3	14.39	0.064	0.411	0.556
N in milk (g/d)	136.3	123.8	138.4	137.1	5.23	0.060	0.088	0.159
N retained (g/d)[4]	25.5	21.0	6.4	10.9	26.75	0.112	0.815	1.000
Energy in milk (kJ/kg of $BW^{0.75}$ per day)	692	625	657	678	32.7	0.776	0.390	0.072
Energy retention total[5] (kJ/kg of $BW^{0.75}$ per day)	80	104	12	25	85.7	0.243	0.763	0.932
Energy retention protein[6] (kJ/kg of $BW^{0.75}$ per day)	29	25	−9	−14	31.8	0.110	0.838	0.990

[1]n = 5 for SL, SH and RH, and n = 4 for RL. SL, diet containing 270 g of slowly fermentable starch per kilogram of concentrate DM; SH, diet containing 530 g of slowly fermentable starch per kilogram of concentrate DM; RL, diet containing 270 g of rapidly fermentable starch per kilogram of concentrate DM; RH, diet containing 530 g of rapidly fermentable starch per kilogram of concentrate DM.

[2]SED, standard error of the difference of means.

[3]ME intake = Gross energy intake – methane production – energy in manure.

[4]N retained = N intake – N in manure – N in milk – N in condensate collected from heat exchanger – N trapped from the outflowing air.

[5]Energy retention total = ME intake – heat production – energy in milk.

[6]Energy retention protein = protein gain × 23.7 kJ/g of protein.

DISCUSSION

This study investigated the effects of starch varying in rate of fermentation and level of inclusion in the diet in exchange with fibre on CH_4 production in lactating dairy cows. Our hypothesis was that increasing the inclusion of ruminally fermentable starch in the diet at the expense of fibre in the diet would increase propionate in the rumen, and that decreasing rate of fermentation starch would shift digestion from the rumen to the small intestine, both expected to decrease CH_4 production expressed per unit of feed or milk. Rumen propionate molar proportion was higher with R starch (16.5 mol/100 mol) than with S starch (15.8 mol/100 mol), but level of starch did not influence rumen molar proportion of propionate. The in situ ruminal starch degradation rates of the two starch sources differed almost by a factor of three (0.054 vs. 0.155 /h; S vs. R starch), and the estimated amount of rumen degraded starch was 508 versus 806 g/kg of dietary starch for S versus R starch. The in situ characteristics were in agreement with our aim to select the starch sources those represent a wide range of starch fermentation rate. Methane expressed per unit of eRFOM was 10% lower for R than S starch and 8% lower for H- than L-based diets. However, CH_4 production per kilogram of milk, per kilogram of FPCM, per kilogram of DMI, or as percentage of GE intake was not influenced by dietary treatments.

Effects on ruminal pH and concentration of VFA

In the current study, the contrast in starch content of the diet was achieved by replacement of fibre-rich beet pulp and palm kernel expeller with maize grain. Much of the non-fibre carbohydrate in beet pulp is pectin and has a tendency for a rumen fermentation profile with more acetate and butyrate (Voelker and Allen, 2003a). Similarly, in a study in which barley was partially substituted with beet pulp in the concentrate, molar proportion of acetate increased and that of propionate and total VFA concentration in the rumen contents decreased (Bodas et al., 2007). Bannink et al. (2006) analysed VFA data from 182 diets and found higher molar proportions of propionic acid to occur upon fermentation of starch compared with fermentation of cellulose or hemicellulose. In contrast with these findings, rumen acetate and propionate molar proportions were not affected by level of starch, although the source of starch (R vs. S) did significantly affect propionate molar proportion. The relatively high mean daily rumen pH recorded with all treatments may have been the reason for the absence of a substantial response in propionic acid production. Bannink et al. (2008) estimated that with elevated rumen pH the fraction of starch fermented to propionic acid

rather than acetic and butyric acid decreases, with the minimum reached at pH values above 6.5. At this pH, the minimum fraction of starch fermented to propionic acid is just slightly higher than the fraction of cellulose or hemicellulose fermented to propionic acid on roughage-type diets (more than 50% roughage on DM basis).

In the present study, starch content increased from on average 116 (low starch level) to 212 (high starch level) g/kg of DM. In a study with diets containing 152, 192, 218, and 224 g/kg of DM of starch from oats, barley, maize, and wheat as the primary source of carbohydrate, respectively, ruminal pH and VFA concentrations were unaffected (Gozho and Mutsvangwa, 2008). In another study, substitution of beet pulp for high-moisture maize up to 243 g/kg of DM also did not affect daily mean or minimum ruminal pH (Voelker and Allen, 2003a). In the present study, the bicarbonate added as a buffer to the high starch concentrates may have prevented a pronounced drop in rumen pH, which is expected to increase rumen propionate proportions (Bannink et al., 2008). This may have contributed to the absence of effect of level of starch on VFA molar proportions in the rumen.

Effects on DMI, nutrient digestibility and milk production

Cows fed H starch-based diets consumed less feed compared with those fed L starch-based diets, whereas source of starch did not affect DMI. The effects of source and level of starch on feed intake are inconsistent among studies reported in literature. In agreement with our results, Miron et al. (2004) reported a reduced DMI on a high-starch concentrate diet compared with a high-NDF concentrate diet. Conversely, in other experiments the type of carbohydrate in concentrate mixture (starch vs. cell wall constituents) in total mixed diets did not affect DMI (De Visser et al., 1990; Abrahamse et al., 2008), whereas Beckman and Weiss (2005) reported a tendency of higher DMI as NDF in the diet increased and starch decreased. Other studies reported an increased DMI by cows fed slowly fermentable maize starch compared to rapidly fermentable barley starch (Casper and Schingoethe, 1989; McCarthy et al., 1989). Intake can be affected by numerous factors such as rate of fermentation of starch and fibre, meal patterns, metabolic fuel absorbed, and ruminal patterns of fermentation and pH (Allen, 2000; Voelker and Allen, 2003b; Reynolds, 2006). In the present study, the ERD of OM in the rumen was 13% higher for cows fed the H starch diet compared with L starch diet, and this higher ERD may be associated with reduced feed intake. With high starch diets resulting in high propionic acid production in the rumen, DMI may decrease due to the hepatic oxidation of propionate affecting feed intake (Allen et al., 2009). Propionate uptake

by liver could have been altered, but was not measured. The supply could have been changed as suggested by Sutton et al. (2003).

A significantly higher apparent total-tract starch digestibility for R starch compared with S starch diets was consistent with rumen ERD of starch. However, the difference in total-tract digestibility was much smaller (972 vs. 955 g of digested starch per kilogram of dietary starch; R vs. S starch based-diets) than the difference in ERD of starch (806 vs. 508 g of rumen-degraded starch per kilogram of dietary starch; R vs. S starch-based concentrates). These values indicate that a much higher fraction of dietary starch could have been digested post-ruminally with S starch than with R starch diets. In agreement with our results, Gozho and Mutsvangwa (2008) reported apparent total-tract digestibility of starch in cows fed rapidly fermentable starch (oats-based diet) to be higher than in cows fed slowly fermentable starch (maize- and wheat-based diets), with no differences observed for DM, OM, and NDF digestibility. In contrast, Ferraretto et al. (2013) in their meta-analysis reported that increased dietary starch levels typically decreased ruminal and total-tract NDF digestibility when cows are fed high-starch diets. The potential for the negative associative effects of high level of fermentable starch on ruminal fibre digestion (Firkins et al., 200; Ferraretto et al., 2013) probably did not occur in the present study with pH remaining at levels high enough not to impair activity of fibrolytic bacteria.

Milk characteristics were not affected by dietary treatments. The lack of effect of starch source on milk fat in the present study is in agreement with Silveira et al. (2007), when cows were fed on wheat-, barley-, or maize-based diets. In contrast, Voelker and Allen (2003b) observed a reduced FCM yield and fat yield when lactating dairy cows were fed a diet with 18% starch compared with 27 and 31% starch diets. The tendency in higher milk protein yield observed with R starch based diets may be explained by the higher eRFOM, because a rise in OM degraded may stimulate microbial protein synthesis leading to an increased absorption of amino acids in the intestine.

Effects on methane production

Based on differences in propionate molar proportion between treatments, a reduction in CH_4 production could be expected with R starch compared with S starch, because high propionic acid levels are associated with reduced methanogenesis (Benchaar et al., 2001). However, in the present study the treatment differences for propionate molar proportion were either not present (level of starch) or rather small in size (source of starch), and this may have contributed to the absence of reduction of CH_4 production per kilogram of DMI, GE intake or

kilogram of FPCM. In contrast, Beauchemin and McGinn (2005) reported lower CH_4 emissions per kilogram of DMI and as a percentage of GE for cattle fed maize compared with barley. Rapidly fermentable starch showed a tendency to decrease the acetate:propionate ratio. However, Martin et al. (2010) suggested that low acetate:propionate ratio and depressed CH_4 production may not always be linked in high-concentrate fed animals. One of the possible explanations for the lack of relationship between CH_4 emission and rumen VFA pattern may be due, in part, to the observation that the molar proportions of ruminal VFA do not necessarily reflect the proportion in which they are produced (Sutton et al., 2003), but rather reflect the balance between production and absorption (Dijkstra et al., 1993). This balance can be influenced by many factors including level of DMI, rumen volume, rumen absorptive capacity and rumen pH (Bannink et al., 2008). In addition, all dietary treatments in the current study were rather high in NDF (378 to 441 g/kg of DM), which could promote chewing, increase salivation rate and have contributed to rumen buffering.

In contrast to the absence of effects of dietary treatments on CH_4 expressed per kilogram of DMI, CH_4 production expressed per unit of eRFOM was lower for R than S starch-based diets. Such an effect was not established when CH_4 production was expressed per kilogram of digested OM, or per kilogram of FPCM, in agreement with data from other studies (Mills et al., 1999; Firkins et al., 2001) because large differences in rumen ED of starch were almost completely compensated by digestion of bypass starch in the intestine. In partial agreement with our results, Hassanat et al. (2013) also found no effect on CH_4 production per unit of feed intake, GE intake, and milk upon increasing dietary starch content from 170 to 228 g/kg of DM in dairy cattle, but a further increase to 300 g/kg of DM did reduce CH_4 production.

This might be an indication that higher starch levels than the highest level of 212 g/kg of DM tested in the present study are required to reduce enteric CH_4 production. In a recent review on CH_4 mitigation options, Hristov et al. (2013) concluded that inclusion of starch rich concentrates below 350 to 400 g/kg of the total diet DM influences CH_4 production to a minor extent only and CH_4 emission intensity decreased particularly with levels above 400 g/kg of DM. Because the starch content in the present study remained well below 400 g/kg of DM and the contrast tested was relatively small (approximately 100 g/kg of DM), the absence of starch level on CH_4 production per kilogram of DMI or per kilogram of FPCM is in line with these findings.

Daily CH_4 production (g/d) was reduced mainly due to a higher level of starch and associated lower DMI, but not due to source of starch in the diet. Mc Geough et al. (2010)

also observed similar responses in daily CH4 output in beef cattle fed whole-crop wheat silages with increasing dietary starch content. The 8% reduction in daily CH_4 production due to high dietary level of starch observed in the present study though was for a large part due to the 4% lower DMI rather than due to the level of starch in the diet. Studies reported in literature confirm the direct relationship between CH_4 production and DMI (Benchaar et al., 2001; Hristov et al., 2013).

Effects on N balance

For better assessment of the overall environmental impact of including different sources and levels of starch in the diet of dairy cows to reduce enteric CH_4 production, emissions of other greenhouse gases such as N_2O need to be accounted for. The amount and form of excreted N has a major impact on emissions of N_2O (Dijkstra et al., 2011), and therefore in the present study we also evaluated the effects of the dietary treatments on N balance. Daily N intake and N retention remained unaffected by treatments. The tendency of higher N output in milk in cows fed R starch-based diets suggests that rumen digested starch was stimulatory for milk protein synthesis and was associated with a numerical net mobilization of N, compared to a numerical net retention of N with S starch-based diets that have been digested relatively more in the small intestine (Mills et al., 2001). In contrast, Ferraretto et al. (2013) reported a tendency of lower N secreted in milk in cows fed rapidly fermentable starch such as wheat-based diet compared with cows fed slowly fermentable starch sources such as barley- or maize- based diets.

The positive N retention on S starch-based diets, and the small negative N retention on R starch-based diets was not in line with the actual BW change recorded (-0.6 vs. -1.3 kg/d, S vs. R). Inherent errors associated with N balance studies in lactating dairy cows (such as volatile N losses from manure during collection) likely result in overestimating the true N retention (as reviewed by Spanghero and Kowalski, 1997; Benchaar et al., 2013; Spek et al., 2013). However, in our study, all major N sources emitted were captured with the setup of our respiration chambers. In addition, across all treatments the N retention was quite close to zero, indicating no such problems of losses has occurred, whereas in a review on dairy cattle N balance trials, Spanghero and Kowalski (1997) calculated an average N balance of 39 g/d.

CONCLUSIONS

Results from this study show that both increasing the level of starch in the diet at the expense of fibre (beet pulp and palm kernel expeller) and increasing the rate of fermentation

of starch did not affect CH_4 emissions expressed per unit of DMI, per unit of OM digested, per unit of GE intake, or per unit of milk produced but does reduce enteric CH_4 emissions of dairy cattle expressed per unit of eRFOM. Rapidly fermentable starch, but not starch level in the diet, increased the propionate molar proportion and tended to decrease the acetate: propionate ratio in the rumen.

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CHAPTER 3

Relationship between in vitro and in vivo methane production measured simultaneously with different dietary starch sources and starch levels in dairy cattle

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ABSTRACT

To investigate the relationship between in vitro and in vivo methane (CH_4) production measured simultaneously using the same rumen-fistulated cows in both experiments, four dietary treatments based on concentrate that accounted for 400 g/kg of the mixed diet dry matter (DM), were formulated to contain starch varying in rate of fermentation (slowly (S) vs. rapidly (R): native vs. gelatinized maize grain) and level of inclusion (low (L) vs. high (H): 270 vs. 530 g/kg of concentrate DM). Sixteen rumen-fistulated lactating dairy cows were used in a complete randomized block design with these treatments replicated in four periods of 17 d each. In experiment 1, after 12 d of adaptation, the cows were housed in respiration chambers for 5 d to measure CH_4 production. In experiment 2, in each period in vitro gas and CH_4 production were measured (in duplicate per period) for mixed diet samples from the same diet as fed to the donor cows using rumen inocula adapted to the respective diets for an average of 16 d. In addition, samples of two concentrate ingredients, viz. grass silage and beet pulp, were incubated with four different inocula obtained from individual donor cows. Gas production (GP) was measured using automated GP system with CH_4 measured at distinct time points. In vitro (24-h) CH_4 production of mixed diet was lower with R than S (42.9 vs. 49.5 ml/g of incubated organic matter (OM); $P = 0.004$), and higher with L than H (49.8 vs. 42.6 ml/g of incubated OM; $P = 0.002$). A significant interaction effect between source and level of starch ($P = 0.015$) was also found, indicating the CH_4 production of the RH diet decreased in particular. In vivo, an increased rate of starch fermentation resulted in a lower CH_4 per unit of estimated rumen-fermentable OM (eRFOM; 55.6 vs. 61.2 ml/g of eRFOM; $P = 0.007$), and higher level of starch tended ($P = 0.089$) to reduce CH_4 per unit of eRFOM, but dietary starch level and source did not affect CH_4 per unit of OM consumed. Across the diets tested, 24-h in vitro CH_4 (ml/g of incubated OM) correlated well with in vivo CH_4 expressed per unit of eRFOM ($R^2 = 0.54$; $P = 0.040$), but not when expressed per unit of OM ingested ($R^2 = 0.04$; $P = 0.878$). For grass silage (the same trend for beet pulp), inocula adapted to R- and H-based diets compared with S- and L-based diets resulted in a lower CH_4 production (36.1 vs. 44.8 ml/g of incubated OM, R vs. S; and 37.4 vs. 43.4 ml/g of incubated OM, H vs. L; $P < 0.001$). These results indicate that adaptation of rumen inoculum to different diets affects CH_4 production of a substrate differently. In conclusion, in vitro CH_4 measurement can be indicative of the trend of in vivo CH_4 production from different combinations of sources and levels of starch when in vivo CH_4 is expressed per unit of eRFOM, but not when expressed per unit of OM ingested. This study suggests that complexity associated with

rumen fermentation conditions needs to be considered to fully predict in vivo CH_4 production from in vitro measurements.

Key words: in vitro; in vivo; starch fermentability; methane; dairy cow

INTRODUCTION

The in vitro gas production (GP) technique is a relatively cheap, well-standardized and widely used method to evaluate the nutritive value of ruminant feeds by incubating substrate in buffered rumen fluid (Cone et al., 1996; Getachew et al., 1998; Dijkstra et al., 2005). This technique can also be used to estimate the methane (CH_4) production potential of different feeds and the CH_4 mitigation potential of feed additives and plant extracts (Getachew et al., 2005; Pellikaan et al., 2011a,b; Bhatta et al., 2012). A number of factors with considerable influence on the results obtained with this technique have been reported (as reviewed by Rymer et al., 2005). Amongst the largest sources of variation are the source and activity of the microbial inoculum (Cone et al., 2002). In most in vitro studies, the microbial inoculum is obtained from donor animals fed a diet, which differs from the substrate used for the in vitro incubation. Under such conditions, the fermentation characteristics of the substrate and the amount of CH_4 produced were shown to be dependent on the type of available substrate in the diet of the donor animal and its fermentation characteristics (Martinez et al., 2010), due to diet-dependent changes in the microbial type and activity of the rumen inoculum (Fernando et al., 2010; Boguhn et al., 2013).

Feeding high levels of rumen fermentable starch or high concentrate diets were observed to change the type of ruminal micro-flora, including increased amylolytic bacteria and decreased methanogen and fibrolytic bacteria numbers (Martin et al., 2010; Morgavi et al., 2010). Similarly, in contrast to fibre, starch fermentation in the rumen was shown to favour the production of propionate (Bannink et al., 2008), creating an alternative hydrogen sink to methanogenesis. However, there is substantial variation in the in vitro fermentation kinetics of different starch sources (Cone and Becker, 2012). Inclusion of these starch sources in the diets of animals also influences rumen fermentation differently, and hence leads to variability in the reduction of CH_4 produced (Beauchemin and McGinn, 2005; Popova et al., 2013). Above all, in vitro and in vivo studies are usually performed separately under different conditions and there is a lack of direct in vitro–in vivo comparison, which is essential to demonstrate the robustness or effectiveness of the in vitro GP technique in simulating rumen fermentation.

Hindle et al. (2005) compared the in vitro and in vivo starch degradation from different sources and found a discrepancy between the in vivo starch degradation and that estimated from an in vitro GP experiment in which the donor animals were not adapted to the diets with the same starch source. The authors suggested in vitro GP could provide a more accurate simulation of the in vivo fermentation of potato starch if the donor animals are adapted to a

diet including this starch source. Cone and Van Gelder (2006) observed that the fermentation rate of native potato starch was considerably enhanced by using rumen fluid of cows adapted to the fermentation of native potato starch, instead of using other rumen fluid of cows fed no potato starch. Although different laboratories around the world are using in vitro GP as a well standardized techniques for the evaluation of the nutritional quality of ruminant feeds, studies that have investigated the relationship between the in vitro and in vivo CH_4 production using the same adapted animals for the in vivo experiment as for collecting rumen inoculum for the in vitro incubations are largely lacking. In an earlier study, Bhatta et al. (2008) found a weak relationship between in vitro CH_4 production (ml/g of DM) estimated by gas production technique with rumen inocula obtained from non-adapted dairy cows and in vivo CH_4 production (ml/g DM intake) by goats fed different diets and measured in respiration chambers.

The objective of this study was to investigate the relationship between in vitro and in vivo CH_4 production measured simultaneously using the same rumen-fistulated dairy cows in both experiments and with the same mixed diet incubated as substrate in vitro and fed to the donor animal of which microbial inoculum is obtained, with the concentrate component of the mixed diet varies in starch rate of fermentation and level of starch. We hypothesized that in vitro CH_4 measurements from different sources and levels of starch in the diet are related to the in vivo CH_4 production if both in vitro and in vivo CH_4 measurements are performed simultaneously, using the same animals as donor for microbial inoculum when they are fed and adapted to the same dietary material used as a substrate for in vitro incubation.

MATERIALS AND METHODS

Experimental design, animals and diets

This study consisted of two experiments conducted simultaneously using the same cows. The experiments were conducted at the animal research facility of Wageningen University (Wageningen, The Netherlands). All experimental procedures were approved by the Institutional Animal Care and Use Committee of Wageningen University and carried out under the Dutch Law on Animal Experimentation.

A total of 16 multiparous lactating Holstein−Friesian dairy cows, all fitted with a permanent rumen cannula were used. Cows were grouped in four blocks using a complete randomized block design based on parity (2.8 ± 1.0; mean ± SD), days in milking (302.4 ± 74.0 d) and fat- and protein-corrected milk (27.1 ± 8.3 kg/d) at the start of the experiment.

Cows within a block were randomly assigned to one of the four different dietary treatments that were based on concentrates formulated to contain starch varying in rate of fermentation (slowly (S) vs. rapidly (R): native vs. gelatinized maize grain, respectively) and level of inclusion (low (L) vs. high (H): 270 vs. 530 g per kg of concentrate DM, respectively). Cows received diets consisting of 600 g/kg of grass silage and 400 g/kg of concentrate (DM basis). The diets were offered individually and in equal meals twice daily during milking at 0600 and 1600 h. The concentrates were in a meal form and mixed with the forage portion manually when fed. Cows had unrestricted access to drinking water. The 16 rumen fistulated cows were part of a larger experiment to investigate effect of starch level and starch source on CH_4 production including 24 non-fistulated animals (Hatew et al., 2015).

The two starch sources were selected to create a considerable contrast in starch rate of fermentation. Different levels of starch in the concentrates were achieved by exchanging either native or gelatinized maize grain with fibrous material (beet pulp and palm kernel expeller) on a DM basis. The ingredient composition of the concentrates was reported by Hatew et al. (2015). The main starch source (i.e. maize grain) was supplied by Meneba Meel BV (Rotterdam, The Netherlands). Gelatinization of starch was performed at 80^{o}C for 2 min using an Insta-Pro extruder (Urbandale, IA, USA). The degree of gelatinization was tested by enzymatic degradation, using an amyloglucosidase test. Gelatinized maize was prepared from the same batch as native maize to avoid differences in chemical composition, as would have been the case when using other rapidly fermentable starch sources, such as oats or wheat, instead of gelatinized maize.

Experiment 1 involved the measurement of in vivo CH_4 emission from cows fed those four dietary treatments replicated in four periods of 17 d each. The first 12 d of each period was used for adaptation to the diet. During the first 8 d of the adaptation period, the cows were fed ad libitum. From day 9 onwards, the cows were fed restricted to 95% of the average daily voluntary DM intake based on day 3–8 of the cow consuming the lowest amount of feed in that particular block to avoid the confounding effects of DM intake on CH_4 production. After the end of the adaptation period, cows were housed for 5 d in one of the two identical climate-controlled open circuit indirect calorimetry respiration chambers for the measurement of CH_4 production. Details of the respiration chambers and gas analysis have been described by Verstegen et al. (1987). For welfare reasons, two cows (one rumen fistulated and the other without cannula) receiving the same treatment were housed in one chamber.

Experiment 2 consisted of in vitro CH_4 measurements using the same four substrates from a sample of the mixed diet as fed to the individual donor cows. This experiment was conducted simultaneously with experiment 1 and, therefore, was replicated over four periods (n = 4 per treatment). In each period, six additional substrates (viz. the four concentrates, grass silage and beet pulp) were incubated with diet-adapted rumen inocula.

In vitro gas production system

Gas production (GP) profiles of the substrates were measured using a fully automated time related GP system (Cone et al., 1996). Samples of each substrate were freeze-dried and ground over a 1-mm sieve using a Wiley mill (Peppink 100AN, Olst, The Netherlands). Approximately 0.5 g (DM basis) of each substrate was weighed into 250 ml fermentation bottles (Schott, Mainz, Germany). Each substrate was weighed in duplicate bottles within each period and replicated on four separate periods on different days.

In total, 10 different substrates were evaluated with four substrates from a sample of the mixed diet as fed to the individual donor cows, another four substrates from a sample of the concentrate included in those diets, and a sample of the grass silage and the beet pulp (Table 1). The concentrates were: 1) concentrate composed of the low level of S starch (270 g of S starch per kg of concentrate DM; SL), 2) concentrate composed of the high level of S starch (530 g of S starch per kg of concentrate DM; SH), 3) concentrate composed of the low level of R starch (270 g of R starch per kg of concentrate DM; RL), and 4) concentrate composed of the high level of R starch (530 g of R starch per kg of concentrate DM; RH). Beet pulp and grass silage were included to investigate the effects of diet-adapted rumen inocula on in vitro CH_4 production of those non-starch substrates. Substrates from the sample of mixed diets and from the concentrates included in those diets were incubated with rumen inoculum obtained from individual donor cows that were adapted to that particular mixed diet and diet consisting of that specific concentrate, respectively. However, beet pulp and grass silage substrates were each incubated with four different rumen inocula obtained from individual cows adapted to each of the four mixed diets.

In each period, rumen fluid was obtained from each of the four individual donor cows that were adapted to one of the four mixed diets on average for 16 d. Equal volumes of rumen fluid (approximately 200−250 ml) were collected from the front ventral, middle ventral and cranial dorsal sac of the rumen of individual donor cows. Rumen fluid from each cow was collected before the morning feeding in a separate insulated flask previously prewarmed and flushed with CO_2. All handlings of the rumen fluid were as described by Cone et al. (1996).

Table 1. Chemical composition of substrate and total mixed diet.

Item	DM (g/kg as fed)	Ash	CP	NDF	ADF	Crude fat	Starch	Sugar	GE[4]
		(g/kg of DM)							
Substrate									
Beet pulp	913	74	89	416	242	10	7	77	16.8
Grass silage	512	88	148	528	296	38	NA[3]	79	19.1
Concentrate[1]									
SL	889	54	169	311	187	43	275	36	18.5
SH	896	50	170	170	90	34	518	22	18.4
RL	890	47	167	308	181	47	303	33	18.7
RH	883	48	186	153	80	41	542	22	18.3
Total mixed diet[2]									
SL	663	74	156	441	252	40	110	62	18.8
SH	666	72	157	385	214	36	207	56	18.8
RL	663	71	156	440	250	42	121	61	18.9
RH	660	72	163	378	210	39	217	56	18.7

[1]SL and SH are concentrates containing 270 and 530 g of slowly fermentable starch per kilogram of concentrate DM, respectively; RL and RH are concentrates containing 270 and 530 g of rapidly fermentable starch per kilogram of concentrate DM, respectively.
[2]Composed of 600 g grass silage per kilogram and 400 g of respective concentrate per kilogram (DM basis).
[3]NA, starch was not analysed in the grass silage.
[4]Gross energy (MJ/kg of DM).

Prior to inoculation, the fermentation bottles were placed in a shaking water bath (Haake SWB25, Clausthal-Zellerfeld, Germany) maintained at 39^{o}C and preflushed with CO_2. The bottles were then inoculated with 60 ml of buffered rumen fluid with a rumen fluid to buffer ratio of 1:2 (v/v) and connected to the fully automated GP apparatus (Cone et al., 1996).

In vitro methane measurement

Methane concentration in the headspace of the fermentation bottle was measured by a gas chromatography (GC; GC8000Top, CE Instruments, Milan, Italy). To allow gas sampling from the headspace, the fermentation bottles were fitted with a side port sealed with a screw cap that is fitted with an air-tight septum (GRACE, XLB-11 Septa 7/16, Breda, The Netherlands) as illustrated by Pellikaan et al. (2011a). At distinct time points (0, 2, 4, 8, 12, 24, 30, 36, 48, 60 and 72 h of incubation), 10 µl aliquots of the bottles headspace gas were sampled through this opening using a gas tight syringe (Gastight® # 1701 Hamilton 1701N,

10 µl Syringe, Point Style 5, Bonaduz, Switzerland) and analysed for CH_4 concentration using GC. The GC was fitted with a flame ionization detector (FID) and stainless steel column (6 m long, 0.53 mm i.d., 25 µm film thicknesses) packed with PoraPack Q 50-80 mesh (GRACE, Breda, The Netherlands). The temperatures of the injector, column and detector were maintained at 150, 60 and 150°C, respectively. The carrier gas was nitrogen, and the pressure for nitrogen, hydrogen and air was set at 100, 50 and 100 kPa, respectively. Peak areas were determined by automatic integration system software for GC (Chrom−Card data system Version 2.4, 2006, Rodano Milan, Italy). The CH_4 concentration in the headspace was determined by external calibration using a certified standard containing a known content of CH_4 (Linde Gas Benelux, Schiedam, The Netherlands).

Curve fitting and calculations

Cumulative gas production data were fitted iteratively to a monophasic Michaelis-Menten equation (Groot et al., 1996; equation (1)) using the non-linear least squares regression procedure in SAS (SAS Institute Inc., 2010).

$$GP = \frac{A}{1 + (B/t)^C} \quad (1)$$

where GP, cumulative amount of gas produced (ml/g of incubated organic matter; OM); A, asymptote gas (ml/g of incubated OM); B, time of incubation at which half of the asymptote gas has been formed ($t_{1/2}$, h); C, the sharpness of the switching characteristic of the profile; t, time (h).

Curve fit parameter estimates of blanks (containing buffered rumen fluid without substrate) were used to correct for gas produced from residual fermentable OM in the inoculum. The cumulative CH_4 production was obtained as follows. Measured CH_4 concentrations were plotted against time and the monophasic sigmoidal model (equation 1) was fitted to the data points. Next, the model parameter estimates were used to compute CH_4 concentrations at each individual valve opening. Cumulative CH_4 production was calculated as the sum of the increased amount of CH_4 between two successive valve openings and the amount of CH_4 vented from the bottle as described by Pellikaan et al. (2011b; equation 2).

$$M = \sum_{i=1}^{n} \{V_{HS}(C_{i+1} - C_i) + G_{i+1}C_{i+1}\} \quad (2)$$

where M, cumulative CH_4 production (ml/g of incubated OM); V_{HS}, bottle headspace volume (ml); C_i and C_{i+1}, CH_4 concentration in the bottle headspace gas at i and i + 1 valve openings,

respectively; G_{i+1}, the amount of gas (ml) vented at i + 1 valve opening; n = total number of valve openings.

The maximum rate of gas or CH_4 production (R_{max}, ml/h) and time at which this maximum is reached (TR_{max}, h) was calculated based on equation 3 and 4, modified from Yang et al. (2005).

$$R_{max} = \frac{A \times B^C \times C \times TR_{max}^{(-C-1)}}{(1 + B^C \times TR_{max}^{-C})^2} \quad (3)$$

$$TR_{max} = B \times [(C\text{-}1) / (C\text{+}1)]^{(1/C)} \quad (4)$$

where A, asymptote gas or CH_4 (ml/g of incubated OM); B, time of incubation at which half of the asymptote gas or CH_4 has been formed ($t_{1/2}$, h); C, the sharpness of the switching characteristic of the profile.

The daily CH_4 production based on in vitro results was calculated using the observed in vivo OM intake of the donor cow multiplied with the 24-h in vitro CH_4 production per unit of incubated OM.

Comparison of in vitro and in vivo methane production

To investigate the relationship between in vitro and in vivo CH_4 production, only data obtained from the four dietary treatments of both experiments were used. Gas produced during the 24 h of incubation period was assumed as a good estimate of ruminal starch disappearance or the extent of fermentation of starch (Bal et al., 2000), and for the correlations between in vitro and in vivo CH_4 production, the 24-h incubation values were used. Correlations were analysed with in vivo CH_4 production expressed in ml per gram of OM consumed, ml per gram of OM digested, and ml per gram of estimated rumen-fermentable organic matter (eRFOM). Details for determination of apparent total-tract digestibility and eRFOM have been reported by Hatew et al. (2015).

Analytical procedures

Diet and substrate samples were freeze-dried, ground using a Wiley mill fitted with a 1-mm sieve and analysed for DM, ash, N, crude fat, starch, sugar, gross energy, neutral detergent fibre (aNDFom; after a pre-treatment with a heat stable amylase and expressed excluding residual ash), and acid detergent fibre (ADFom; expressed excluding residual ash) following the standard procedures previously reported (Hatew et al., 2015).

Statistical analysis

Data from duplicate bottles for each substrate per period were averaged before statistical analysis. The experimental unit for the in vitro measurements was the value of the averaged bottles and for the in vivo measurement, it was a chamber. The data for one cow fed on RL diet in one respiration chamber had to be excluded from all in vivo and in vitro analysis due to malfunctioning of the chamber and yielding unreliable data. Data were analysed by ANOVA using the PROC MIXED procedure in SAS (SAS Institute Inc., 2010) using the model:

$$Y_{ijk} = \mu + S_i + L_j + P_k + (S \times L)_{ij} + e_{ijk}$$

where Y_{ijk}, the response variable (such as CH_4 and gas production, fermentation kinetic parameters); μ, the overall mean; S_i, the fixed effect of source of starch (i = 2, slowly and rapidly fermentable starch sources); L_j, the fixed effect of level of starch (j = 2, low and high level of starch in the diet or concentrate); P_k, experimental period as a random factor (k = 4); $(S \times L)_{ij}$, the interaction of source and level of starch in dietary treatments; e_{ijk}, experimental error.

Because of unequal variances, the Kenward-Roger option was used to estimate the denominator degrees of freedom. For each analysis, the first autoregressive, compound symmetry and unstructured covariance structures were tested. Depending on the characteristics of analysis, the covariance structure with the lowest Akaike's information criterion was selected (Littell et al., 1998), which in most cases was the compound symmetry covariance structure. The choice of covariance structure was based on parameters estimated from the restricted maximum likelihood method. To examine the correlation between in vitro and in vivo CH_4 production, Pearson correlation coefficients were estimated using the CORR procedure of SAS.

All results are reported as least square means. Effect of treatments and their interactions were declared significant at $P \leq 0.05$ and a tendency at $0.05 < P < 0.10$.

RESULTS

Chemical composition of substrates and donor cow diets

The NDF content of the diets varied between 378 and 441 g/kg of DM and starch content between 110 and 217 g/kg of DM (Table 1). Similarly, the concentrates were highly variable in NDF and starch contents. The crude protein content was similar for all diets, except a slightly higher level for the RH diet. Among the substrates investigated, the NDF content of grass silage was higher compared to the other substrates.

In vitro gas and methane production of dietary treatments

Gas production (GP), CH_4 production and fermentation kinetics of concentrates incubated with rumen inoculum obtained from donor cows adapted to the diet including that specific concentrate are summarized in Table 2, and those of the mixed diet are presented in Table 3. With incubation of the concentrates, an increasing rate of starch fermentation gave a decreased 24-h GP (294.7 vs. 328.6 ml/g of incubated OM, R vs. S; $P = 0.010$). Similarly, lower asymptote GP (343.1 vs. 390.1 ml/g of incubated OM; $P = 0.022$) and CH_4 production (50.4 vs. 58.7 ml/g of incubated OM; $P = 0.006$) were observed with R- compared with S-based concentrates. The half time of GP showed only a tendency ($P = 0.054$) to be lower for R than for S. The level of starch did not affect both GP and asymptote GP, but H compared with L in the concentrate decreased CH_4 production (51.9 vs. 57.2 ml/g of incubated OM; $P = 0.041$).

The asymptote CH_4 production was lower for R- compared with S-based concentrates (70.7 vs. 80.4 ml/g of incubated OM; $P = 0.014$). The TR_{max} for both GP and CH_4 production and B for CH_4 production were unaffected by source and level of starch in the concentrate. However, the R_{max} of GP was lower ($P = 0.044$) for H than L starch in the concentrate. The R_{max} of CH_4 production was reduced ($P = 0.045$) for R compared with S and showed a tendency ($P = 0.095$) to be lower for H than for L. The proportion of CH_4 in the total gas varied between 16.3% for RH and 18.1% for SL concentrate, and was less ($P = 0.028$) for H than for L but unaffected by the source of starch in the concentrate.

The in vitro gas and CH_4 production of diets as fed to the donor cows is presented in Table 3. Diets composed of R compared with S had lower 24-h gas (264.9 vs. 290.7 ml/g of incubated OM; $P = 0.001$) and asymptote GP (296.5 vs. 331.9 ml/g of incubated OM; $P < 0.001$). The higher level of starch in the diet tended ($P = 0.094$) to reduce GP, but did not affect asymptote GP. A significant interaction between source and level of starch indicated that the effects on 24-h GP and asymptote GP were not additive. Lower CH_4 production (42.9 vs. 49.5 ml/g of incubated OM; $P = 0.004$) and asymptote CH_4 production (57.7 vs. 70.4 ml/g of incubated OM; $P = 0.002$) were observed for the diets based on R compared with S. Higher starch inclusion compared with low starch in the diet reduced CH_4 production (42.6 vs. 49.8 ml/g of incubated OM; $P = 0.002$), but asymptote CH_4 production was unaffected by the higher level of starch in the diet. A significant interaction effect of source and level of starch on CH_4 production was also found.

Table 2. In vitro gas and methane production of concentrate substrates incubated with diet-adapted rumen inocula, with the diets of the donor cows varying in starch rate of fermentation and level of inclusion.

	Concentrate[1]					*P*-value		
Item[3]	SL	SH	RL	RH	SED[2]	Source (S)	Level (L)	S × L
Gas production (GP)								
24-h GP (ml/g of incubated OM)	329.6	327.6	311.2	278.2	14.92	0.010	0.130	0.174
A (ml/g of incubated OM)	390.9	389.2	362.5	323.7	24.12	0.022	0.266	0.305
B (h)	7.2	7.6	6.1	6.8	0.61	0.054	0.198	0.794
C	1.6	1.7	1.6	1.7	0.23	0.710	0.512	0.921
R_{max} (ml/h)	34.8	33.4	37.2	30.6	2.42	0.905	0.044	0.163
TR_{max} (h)	2.9	3.5	2.2	3.0	1.01	0.451	0.359	0.891
CH_4 production								
24-h CH_4 (ml/g of incubated OM)	60.4	56.9	54.0	46.8	3.13	0.006	0.041	0.425
A (ml/g of incubated OM)	82.0	78.8	73.7	67.7	4.36	0.014	0.167	0.658
B (h)	11.1	12.0	11.1	12.5	1.18	0.742	0.183	0.737
C	1.5	1.5	1.4	1.4	0.19	0.559	0.986	0.929
R_{max} (ml/h)	4.4	4.1	3.9	3.0	0.49	0.045	0.095	0.420
TR_{max} (h)	3.5	3.8	3.2	3.3	1.36	0.712	0.815	0.956
CH_4 (% of total gas)	18.1	17.1	17.0	16.3	0.57	0.106	0.028	0.420

[1]Sample from the same concentrate as fed to the donor cows was incubated with inoculum adapted to the respective concentrate included in the mixed diet of the individual donor cow. SL and SH are concentrates containing 270 and 530 g of slowly fermentable starch per kilogram of concentrate DM, respectively; RL and RH are concentrates containing 270 and 530 g of rapidly fermentable starch per kilogram of concentrate DM, respectively.

[2]SED, standard error of the difference of means.

[3]A, asymptote gas or CH_4 production; B, incubation at which time half of asymptote gas or CH_4 production has been formed; C, the sharpness of the switching characteristic for the profile; R_{max}, maximum gas or CH_4 production rate; TR_{max}, time occurrence of R_{max}.

Dietary treatments had no effect on R_{max} of GP and TR_{max} of both GP and CH_4 production (Table 3). However, a significant interaction effect of source and level of starch in the diet on R_{max} of CH_4 production was observed, indicating that the effect of source of starch depend on level of starch and vice versa. Half-time GP was unaffected by treatments, with only a tendency (P = 0.086) of higher half-time for CH_4 production with H (14.3 h) compared with L (11.4 h). When the amount of CH_4 was related to total gas (% of total gas), the

Table 3. In vitro gas and methane production of diet substrates incubated with diet-adapted rumen inocula, with the diets of the donor cows varying in starch rate of fermentation and level of inclusion.

	Diet[1]					*P*-value		
Item[3]	SL	SH	RL	RH	SED[2]	Source (S)	Level (L)	S × L
Gas production (GP)								
24-h GP (ml/g of incubated OM)	284.4	297.0	280.8	249.0	7.19	0.001	0.094	0.002
A (ml/g of incubated OM)	325.1	338.7	313.8	279.2	9.17	<0.001	0.143	0.006
B (h)	7.0	7.4	6.7	7.1	0.33	0.192	0.102	0.945
C	1.7	1.7	1.7	1.9	0.12	0.292	0.241	0.625
R_{max} (ml/h)	29.1	29.1	29.7	27.0	1.08	0.357	0.126	0.114
TR_{max} (h)	2.9	3.4	3.0	3.5	0.45	0.852	0.187	0.986
CH_4 production								
24-h CH_4 (ml/g of incubated OM)	50.6	48.4	48.9	36.8	2.33	0.004	0.002	0.015
A (ml/g of incubated OM)	71.5	69.3	58.9	56.4	3.98	0.002	0.425	0.957
B (h)	13.3	13.1	9.4	15.4	2.10	0.608	0.086	0.063
C	1.4	1.4	1.6	1.3	0.12	0.284	0.039	0.126
R_{max} (ml/h)	3.6	3.7	3.9	2.6	0.40	0.206	0.069	0.038
TR_{max} (h)	3.5	3.0	4.0	3.3	0.68	0.447	0.252	0.819
CH_4 (% of total gas)	18.0	16.3	17.2	14.8	0.76	0.051	0.003	0.500

[1]Sample from the same diet as fed to the donor cows was incubated with rumen inoculum obtained from individual donor cow adapted to the respective diet. SL and SH are diets containing 270 and 530 g of slowly fermentable starch per kg of concentrate DM, respectively; RL and RH are diets containing 270 and 530 g of rapidly fermentable starch per kg of concentrate DM, respectively.
[2]SED, standard error of the difference of means.
[3]A, asymptote gas or CH_4 production; B, incubation at which time half of asymptote gas or CH_4 production has been formed; C, the sharpness of the switching characteristic for the profile; R_{max}, maximum gas or CH_4 production rate; TR_{max}, time occurrence of R_{max}.

difference between treatments remained significant (P = 0.003) for H versus L (15.6 vs. 17.6%), and showed a tendency (P = 0.051) to be lower for R- compared with S-based diets.

In vitro and in vivo methane production of dietary treatments

The in vitro and in vivo CH_4 production of diets varying in the rate of starch fermentation and level of inclusion are summarized in Table 4. The 24-h in vitro CH_4 production was less with R than S (42.9 vs. 49.5 ml/g of incubated OM; P = 0.004) and with H than L (42.6 vs.

Table 4. In vitro and in vivo methane production of diets varying in starch rate of fermentation and level of inclusion.

	Diet[1]					*P*-value		
Item	SL	SH	RL	RH	SED	Source (S)	Level (L)	S × L
In vitro CH_4 production								
24-h CH_4 (l/d)[2]	828	751	797	570	39.8	0.005	0.001	0.028
12-h CH_4 (ml/g of OM)	36.4	30.9	34.9	25.8	3.91	0.270	0.029	0.536
24-h CH_4 (ml/g of OM)	50.6	48.4	48.9	36.8	2.33	0.004	0.002	0.015
In vivo CH_4 production[3]								
CH_4 (l/d)	597	545	581	557	27.8	0.916	0.081	0.488
CH_4 (ml/g of OM intake)	36.5	35.6	35.7	36.0	1.31	0.796	0.750	0.518
CH_4 (ml/g of DOM)	46.7	50.7	51.6	47.5	2.79	0.685	0.968	0.075
CH_4 (ml/g of eRFOM)[4]	62.7	59.6	57.0	54.2	2.19	0.007	0.089	0.901

[1]Sample from the same diet as fed to the donor cows was incubated with rumen inoculum obtained from individual donor cow adapted to the respective diet. SL and SH are diets containing 270 and 530 g of slowly fermentable starch per kilogram of concentrate DM, respectively; RL and RH are diets containing 270 and 530 g of rapidly fermentable starch per kilogram of concentrate DM, respectively.
[2]Daily in vitro CH_4 production was calculated using in vivo OM intake of the donor cow multiplied with the 24-h in vitro CH_4 production per unit of incubated OM.
[3]In vivo CH_4 production of donor cows fed on the same diet as substrate incubated was measured simultaneously in climate-controlled respiration chambers.
[4]eRFOM, estimated rumen-fermentable organic matter based on nylon bag degradation characteristics (for details see Hatew et al., 2015).

49.8 ml/g of incubated OM; $P = 0.002$). A significant interaction between source and level of starch on the in vitro CH_4 production indicated that the effect of source of starch was more noticeable with H than L starch in the diet. Treatment differences in 24-h CH_4 production were more pronounced than treatment differences in 12-h CH_4 production. The daily in vitro CH_4 production was lower with a high level compared with a low level of starch in the diet (661 vs. 813 l/d, respectively; $P = 0.005$). Similarly, R compared with S in the diet resulted in a lower daily in vitro CH_4 production (684 vs. 790 l/d; $P = 0.001$). In contrast to daily in vitro CH_4 production, simultaneously measured daily in vivo CH_4 production showed only a tendency ($P = 0.081$) to be lower for R- compared with S-based diets. However, in vivo CH_4 production expressed per unit of eRFOM was affected by starch source (61.2 vs. 55.6 ml/g of eRFOM; S vs. R; $P = 0.007$) and tended ($P = 0.089$) to be affected by starch level (59.9 vs. 56.9 ml/g of eRFOM; L vs. H). However, in vivo CH_4 production expressed per unit of OM intake or per unit of OM digested was unaffected by treatments.

Relationship between in vitro and in vivo methane production

Across the diets tested, 24-h in vitro CH_4 production per unit of incubated OM correlated well with the in vivo CH_4 production expressed per unit of eRFOM (R^2 = 0.54; *P* = 0.040; Figure 1), but not when expressed per unit of OM ingested (R^2 = 0.04; *P* = 0.878; Figure 2) or when expressed per unit of OM digested (R^2 = 0.05; *P* = 0.868; data not shown).

In vitro total gas and methane production of non-starch substrates

Table 5 summarizes the in vitro total gas, CH_4 production and fermentation kinetics of beet pulp and grass silage, each incubated with rumen inocula adapted to four different diets. Inocula adapted to R- and H-based diets compared with S- and L-based diets resulted in a lower 24-h GP of beet pulp (321.6 vs. 342.4 ml/g of incubated OM, R vs. S; *P* = 0.008, and321.9 vs. 342.2, H vs. L; *P* = 0.009) and grass silage (219.8 vs. 241.8 ml/g of incubated OM, R vs. S; *P* < 0.001, and 223.2 vs. 238.4 ml/g of incubated OM, H vs. L; *P* = 0.002). Similarly, 24-h CH_4 production was lower for beet pulp (42.9 vs. 52.5 ml/g of incubated OM; *P* < 0.001) and grass silage (36.1 vs. 44.8 ml/g of incubated OM; *P* < 0.001) when the substrates were incubated with inoculum adapted to R- compared with S-based diets. Increasing the level of starch in the diet of the donor cow decreased the 24-h CH_4 production for both substrates (42.7 vs. 52.7 ml/g of incubated OM, H vs. L for beet pulp; and 37.4 vs. 43.4 ml/g of incubated OM, H vs. L for grass silage; *P* < 0.001). There was also a significant interaction effect of source and level of starch on 24-h GP of grass silage and 24-h CH_4 production for both substrates.

The asymptote GP and CH_4 production of both grass silage and beet pulp was lower for R and H compared to S and L starch in the diet of the donor cow (Table 5). A significant interaction effect of source and level of starch was observed for asymptote GP and asymptote CH_4 production of grass silage, but not for beet pulp. The effects of diet-adapted rumen inoculum on fermentation kinetics, such as R_{max} of GP were similar for beet pulp and grass silage. A lower R_{max} of GP was observed when both substrates were incubated with rumen inoculum adapted to R- and H- compared with S- and L-based diets, with a significant interaction effect of source and level of starch in the diet. Similar results were obtained for R_{max} of CH_4 production of beet pulp and grass silage, except for the lack of an effect of level of starch with grass silage. However, there was a significant interaction effect of source and level of starch in the diet of the donor cow on R_{max} of CH_4 production for grass silage showing that an increased level of R and S resulted in a lower and slightly higher R_{max}, respectively. The half time of GP and CH_4 production was higher for beet pulp only with R-

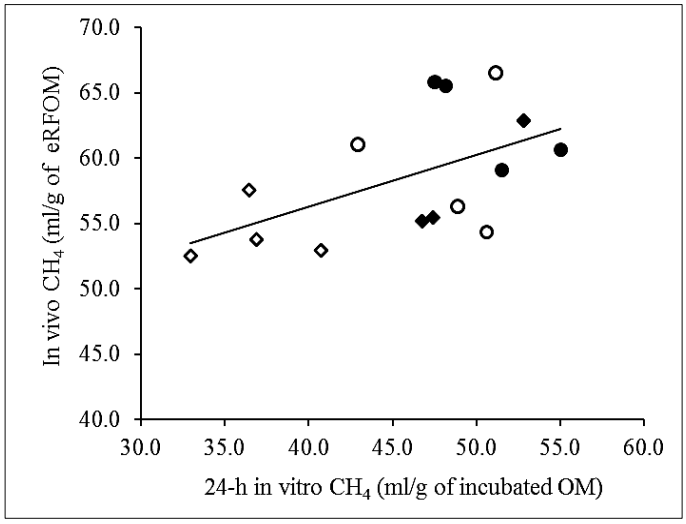

Figure 1. Relationship between in vivo (ml/g of eRFOM) and 24-h in vitro (ml/g of incubated OM) CH_4 production measured simultaneously with dietary starch varying in rate of fermentation and level of inclusion in dairy cows (R^2 = 0.54; *P* = 0.040). Sample from the same diet as fed to the donor cows was incubated with rumen inoculum obtained from individual donor cow adapted to the respective diet. ● = diet containing 270 g of slowly fermentable starch per kilogram of concentrate DM); ○ = diet containing 530 g of slowly fermentable starch per kilogram of concentrate DM); ◆ = diet containing 270 g of rapidly fermentable starch per kilogram of concentrate DM; ◇ = diet containing 530 g of rapidly fermentable starch per kilogram of concentrate DM starch). In vivo CH_4 production of donor cows was measured in climate-controlled respiration chambers.

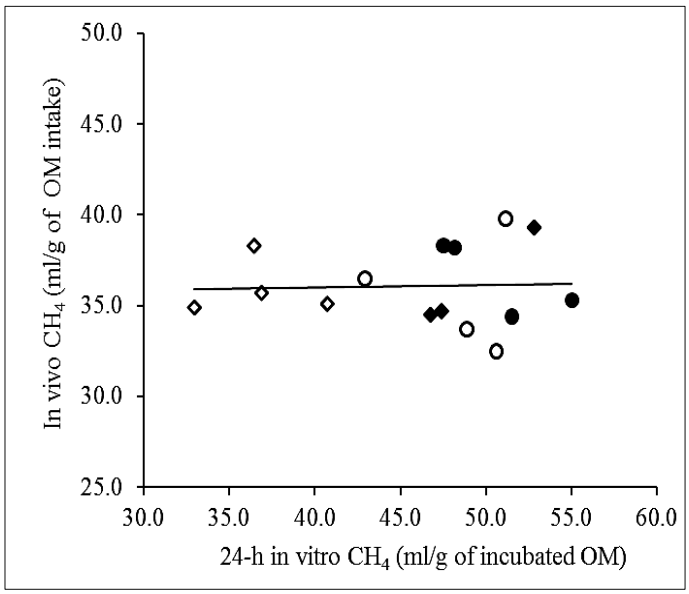

Figure 2. Relationship between in vivo (ml/g of OM intake) and 24-h in vitro (ml/g of incubated OM) CH_4 production measured simultaneously with dietary starch varying in rate of fermentation and level of inclusion in dairy cows (R^2 = 0.04; *P* = 0.878). Sample from the same diet as fed to the donor cows was incubated with rumen inoculum obtained from individual donor cow adapted to the respective diet. ● = diet containing 270 g of slowly fermentable starch per kilogram of concentrate DM); ○ = diet containing 530 g of slowly fermentable starch per kilogram of concentrate DM); ◆ = diet containing 270 g of rapidly fermentable starch per kilogram of concentrate DM; ◇ = diet containing 530 g of rapidly fermentable starch per kilogram of concentrate DM starch). In vivo CH_4 production of donor cows was measured in climate-controlled respiration chambers.

and H- compared with S- and L-based diets, and with an interaction effect of source and level of starch on half time.

The proportion of CH_4 in the total gas when beet pulp was incubated as substrate was reduced by inocula obtained from R- and H- compared with S- and L-based diets, respectively, with an interaction effect of source and level of starch. With grass silage, the proportion of CH_4 in total gas was lower (*P* = 0.011) for R compared with S, and a tendency (*P* = 0.071) for lower CH_4 proportion in H versus L was observed.

Table 5. In vitro gas and methane production of beet pulp and grass silage incubated with diet-adapted rumen inocula.

	Diet[1]					*P*-value		
Parameter[3]	SL	SH	RL	RH	SED[2]	Source (S)	Level (L)	S × L
Gas production (GP)								
Beet pulp								
24-h GP (ml/g of incubated OM)	347.5	337.3	336.8	306.4	8.73	0.008	0.009	0.123
A (ml/g of incubated OM)	381.5	372.1	367.9	341.5	11.18	0.023	0.050	0.313
B (h)	5.6	5.5	5.9	7.6	0.44	0.004	0.020	0.019
C	1.8	1.8	2.0	2.2	0.11	0.013	0.397	0.130
R_{max} (ml/h)	43.7	42.3	41.5	31.0	1.52	<0.001	<0.001	0.002
TR_{max} (h)	2.8	2.7	3.2	4.7	0.48	0.005	0.067	0.037
Grass silage								
24-h GP (ml/g of incubated OM)	243.0	240.6	233.8	205.7	4.84	<0.001	0.002	0.005
A (ml/g of OM)	302.3	295.2	291.2	243.8	7.72	<0.001	0.001	0.005
B (h)	8.4	7.9	8.2	9.1	0.33	0.060	0.452	0.010
C	1.3	1.3	1.3	1.7	0.10	0.017	0.012	0.007
R_{max} (ml/h)	22.7	24.2	22.9	17.2	0.69	<0.001	0.002	<0.001
TR_{max} (h)	2.0	1.7	1.7	4.3	0.53	0.015	0.013	0.004
Methane production								
Beet pulp								
24-h CH_4 (ml/g of incubated OM)	55.4	49.5	50.0	35.8	1.81	<0.001	<0.001	0.010
A (ml/g of incubated OM)	71.2	70.8	70.2	62.5	2.58	0.006	0.009	0.400
B (h)	12.6	13.6	13.5	20.4	1.25	0.002	0.002	0.008
C	1.6	1.4	1.5	1.6	0.13	0.723	0.560	0.260
R_{max} (ml/h)	3.8	3.3	3.3	1.9	0.13	<0.001	<0.001	0.001
TR_{max} (h)	4.8	4.0	4.5	7.6	0.94	0.032	0.105	0.016
CH_4 (% of total gas)	15.9	14.7	14.9	11.7	0.49	<0.001	<0.001	0.020
Grass silage								
24-h CH_4 (ml/g of incubated OM)	45.8	43.7	41.0	31.1	1.40	<0.001	<0.001	0.003
A (ml/g of incubated OM)	68.9	66.1	65.1	44.4	3.88	0.001	0.002	0.010
B (h)	14.3	14.0	15.6	14.0	1.60	0.552	0.417	0.597

Table 5. (continued)

Parameter	Diet[1]				SED[2]	*P*-value		
	SL	SH	RL	RH		Source (S)	Level (L)	S × L
C	1.3	1.2	1.2	1.6	0.09	0.018	0.025	0.002
R_{max} (ml/h)	3.3	3.4	3.0	2.1	0.29	0.005	0.102	0.049
R_{max} (h)	2.7	1.7	2.0	5.0	0.81	0.055	0.110	0.006
CH_4 (% of total gas)	18.9	18.2	17.6	15.9	0.81	0.011	0.071	0.461

[1]Samples from grass silage and beet pulp were each incubated with four different diet-adapted rumen 270 and 530 g of slowly fermentable starch per kilogram of concentrate DM, respectively; RL and RH are diets containing 270 and 530 g of rapidly fermentable starch per kilogram of concentrate DM, respectively.

[2]SED, standard error of the difference of means.

[3]A, asymptote gas or CH_4 production; B, incubation at which time half of asymptote gas or CH_4 production has been formed; C, the switching characteristics for the profile; R_{max}, maximum gas or CH_4 production rate; TR_{max}, time occurrence of R_{max}.

DISCUSSION

To our knowledge, this is the first study where in vitro CH_4 measurements were performed simultaneously with in vivo CH_4 measurements in order to test the potential of an in vitro GP to predict actual CH_4 production in vivo. This was achieved by using adapted animals in an in vivo trial as donor animals for rumen inocula used with the in vitro incubations. The in vitro CH_4 measurements (expressed per unit of OM incubated) at 24-h were shown to be significantly correlated to in vivo rumen CH_4 production determined from different combinations of sources and levels of starch in the diet when in vivo CH_4 production was expressed per unit of eRFOM, but were not correlated when expressed per unit of OM ingested or per unit of OM digested.

Relationship between in vitro and in vivo methane production

Given the variation in chemical composition of the substrates and diets (Table 1) and in situ rumen degradation characteristics of starch (Hatew et al., 2015), the effect of dietary treatments on in vitro CH_4 production followed the expected patterns of starch rate of fermentation and level of inclusion when evaluated either by incubating concentrate substrates (Table 2) or mixed diet substrates (Table 3). The fractional degradation rate of the two starch sources varied almost by a factor of three (0.054 vs. 0.155 /h; S vs. R starch) and the estimated amount of rumen degraded starch was 59% higher for R compared with S (Hatew et al., 2015), which shows the markedly differing rates of fermentation of the two selected starch

sources. Increased fractional rate of starch fermentation and level of inclusion in the diet reduced 24-h in vitro CH_4 production by 14%. In agreement with this, Getachew et al. (2005) observed a higher in vitro CH_4 production for a slowly digestible fraction of the feed (such as structural carbohydrate).

We measured CH_4 production of dairy cattle in respiration chambers and used rumen fluid of the very same individual animals to measure CH_4 production in vitro, allowing in vitro CH_4 productions to be related with the actual rumen CH_4 emissions for individual dietary treatments. The in vivo CH_4 production expressed in ml per gram of eRFOM was 4.9% lower with the SH than with SL the diet (Table 4). This is similar to a 4.3% reduction in 24-h in vitro CH_4 production expressed in ml per gram of incubated OM. However, differences in CH_4 production were found to be much greater in vitro than in vivo when comparing other combinations of starch sources and levels. The 24-h in vitro CH_4 production of the RH diet was 27.3% less compared to the SL diet. This reduction was almost double the difference of 13.6% for in vivo expressed in ml per gram of eRFOM. In agreement with our results, more pronounced differences in CH_4 production between diets observed in vitro than in vivo have been reported previously. Martinez-Fernandez et al. (2013) showed a proportionally higher reduction in CH_4 production in vitro by two plant compounds by as much as 87 and 96% compared with a relatively lower in vivo CH_4 reduction (33 and 64%, respectively) per unit of DM intake in goats. In their study, the in vitro and in vivo experiments were not performed in parallel as done in our study, where we used the same animals from the in vivo experiment as donors for collecting the diet-adapted rumen inocula for the in vitro trial.

The in vitro (24-h) CH_4 production per unit of incubated OM correlated well with the in vivo CH_4 expressed per unit of eRFOM (Figure 1), but not when expressed per unit of OM ingested (Figure 2) or per unit of OM digested (R^2 = 0.05; P = 0.868; data not shown). In contrast, 12-h in vitro CH_4 production was unrelated to in vivo CH_4 production expressed either per unit of eRFOM (R^2 = 0.34; P = 0.210), per unit of OM ingested (R^2 = 0.03; P = 0.908) or per unit of OM digested (R^2 = −0.14; P = 0.631) (data not shown). In vivo OM digestion includes the combined effects of rumen fermentation and large intestine fermentation, may not fully reflect rumen OM fermentation only, whereas the in vitro method only simulates rumen fermentation but not intestinal digestion. Even though both in vitro and in vivo experiments were done simultaneously under the same conditions, the in vitro study still did not take into account the influences of complex and dynamic fermentation conditions

that occurs during the degradation of feeds in the rumen, such as rumen outflow of unfermented material (Pinares-Patino et al., 2007; Bannink et al., 2011; Dijkstra et al., 2012), or changes in rumen pH and buffering capacity (Mc Geough et al., 2011; Dijkstra et al., 2012) that occur under in vivo conditions. The ruminal fluid dilution rate and passage rate of substrate, for instance, were reported to explain about 25 and 28%, respectively, of the variation in CH_4 production in cattle (Okine et al., 1989). The latter is probably due to a reduced retention times in the rumen, and hence decreased rate of substrate fermentation and CH_4 production. The absence of such rumen processes in the closed in vitro GP system used in the current study may explain the absence of a relationship between the in vitro CH_4 expressed per unit of incubated OM and in vivo CH_4 expressed per unit of OM ingested or per unit of OM digested.

The in vivo CH_4 production measured in the current study originated from both rumen and post-ruminal fermentation. Yet the daily in vitro CH_4 production calculated from 24-h in vitro CH_4 production multiplied by the in vivo OM intake is higher than the actually measured daily in vivo CH_4 production for all dietary treatments. A similar result was obtained by Bhatta et al. (2007) who reported a lower in vivo CH_4 production measured by sulphur hexafluoride (SF_6) compared with 48-h in vitro CH_4 (measured by a syringe method) with all diets tested (alfalfa hay, maize silage, Italian ryegrass hay, rice straw and Sudan grass hay). In contrast to the present results, the same authors reported a close correlation between in vitro CH_4 production estimated from the mean of the two measurement intervals (24- and 48-h) and simultaneously measured in vivo CH_4 production for most diets mentioned earlier. The discrepancy between the studies might be due to the difference in the techniques used or due to the variations in the fermentation characteristics of the diets investigated. In the present study, CH_4 produced during the first 24-h accounted for 54 to 88% of the asymptote CH_4 production, and the gas produced at 24 h of incubation was considered to be a good estimate of the extent of starch fermentation (Bal et al., 2000). The in vitro CH_4 production at 12-h of incubation was only 35 to 69% of asymptote CH_4 produced, and did not improve correlations with CH_4 production in vivo, compared with the 24-h in vitro CH_4 production.

The decline in CH_4 produced per unit of incubated OM associated with rumen inoculum adapted to the RH diet might have been caused by a shift towards a more propionate oriented type of fermentation, leading to less CH_4 production. Propionate is a sink for hydrogen and, therefore, hydrogen is unavailable as a substrate for methanogens. The decrease in 24-h in vitro CH_4 production corresponds to the expected relative increase in propionate with R-based

diets with a relatively higher contribution of a rapidly fermentable starch with the higher inclusion level. In a previous study, in vitro fermentation of different sources of starch showed that rapidly rumen fermentable starch sources resulted in a higher molar proportion of propionate (Cone and Becker, 2012). The absence of significant difference in the in vitro GP between L and H starch-based concentrates but a significantly lower CH_4 production for H starch-based concentrates (Table 2) might indicate the contribution of the higher level of starch towards propionate production. This could be due to the fact that one mole of gas is produced per mole of acetate produced, but no net gas is produced in the propionate fermentation pathway (Firkins et al., 1998) and only fermentation to acetate and butyrate produces CO_2 and consequently CH_4 (Blümmel and Ørskov, 1993). However, no ruminal VFA concentration and pH were measured in the present in vitro study and, therefore, the present results are not conclusive.

Effect of diet-adapted rumen inoculum

Adaptation of the rumen inoculum to different sources and amounts of starch affected the CH_4 production from a substrate differently (Table 5). Inoculum obtained from cows adapted to R-based diets resulted in lower CH_4 production for both substrates (grass silage and beet pulp). Consistent with our results, Cone and Van Gelder (2006) observed that the fermentation rate of native potato starch was enhanced by using rumen fluid adapted to the fermentation of native potato starch instead of using other rumen fluids. The lower CH_4 production with inocula adapted to the R-based diets in the present study might be due to a change in a fermentative activity, i.e. an altered microbial composition, a change in microbial enzyme activity, or a combination of both (Fernando et al., 2010; Boguhn et al., 2013). However, no data were collected on microbial dynamics, which might have shown a dependency between the pattern of rumen microbial population and type of diet fed. In line with this, information in the literature shows the extent to which the microbial ecosystem adapts to a particular type of diet. A study by Hristov et al. (2001) reported that rumen protozoa numbers are often lower in cattle fed a high-grain diet with less CH_4 being produced, presumably due to a decreased transfer of hydrogen from protozoa to methanogens. Similarly, feeding high levels of rumen fermentable starch or high concentrate diets have been observed to result in changes in the type of ruminal micro-flora, including increased amylolytic bacterial and decreased methanogens and fibrolytic bacterial numbers (Morgavi et al., 2010). Increasing the ratio of concentrate to hay in the diet of donor animals was shown to reduce the

initial bacterial concentration and to affect the GP kinetic parameters, such as total GP and rate of GP (Nagadi et al., 2000).

Taken together, results from the present study suggest that the complexity of rumen fermentation conditions needs to be taken into consideration in predicting the in vivo CH_4 production from in vitro GP measurements with varying starch sources and levels in the diet. It appears important to consider the diet of the donor animal, since incubation of the same substrate (grass silage or beet pulp) with rumen inocula obtained from donor cows fed on different diets produced variable amount of methane (Table 5). Whether the effects observed in the present study are pertinent to other types of ruminant diets and substrates incubated in vitro requires further investigation.

CONCLUSIONS

The potential of an in vitro gas production to predict actual CH_4 production in vivo was evaluated using the same adapted dairy cows in the in vivo trial as donor animals for rumen inocula used for the in vitro incubations simultaneously. In vitro CH_4 production is correlated with in vivo CH_4 production from different combinations of sources and levels of starch in the diet when in vivo CH_4 production was expressed per unit of eRFOM, but not correlated when expressed per unit of OM ingested or per OM digested.

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CHAPTER 4

Increasing harvest maturity of maize silage reduces methane emission of lactating dairy cows

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ABSTRACT

The objective of this study was to investigate the effects of increasing maturity of whole-plant corn at harvest on methane (CH_4) emission by dairy cows consuming maize silage (MS) based diets. Whole-plant maize was harvested at a very early (25% dry matter (DM); MS25), an early (28% DM; MS28), a medium (32% DM; MS32) and a late (40% DM; MS40) stage of maturity. In a randomized block design 28 lactating Holstein-Friesian dairy cows, of which eight were fitted with rumen-cannula, received one of four dietary treatments designated as T25, T28, T32 and T40 to reflect the DM contents at harvest. Treatments consisted of (DM basis) 75% of each individual MS, 20% concentrate and 5% wheat straw. Concentrate and straw were similar for all the treatments. Feed intake was restricted (95% of ad libitum DM intake) to avoid the confounding effects of DM intake on CH_4 production. Feed intake, digestibility, milk production and composition, energy and nitrogen (N) balance, and CH_4 production were measured during a 5-d period in climate respiration chambers after an adaptation to the diet for 12 d. Maize silage starch content varied between 275 (MS25) and 385 (MS40) g/kg of DM. Treatments did not affect DM intake (DMI; mean 18.0 kg/d), milk yield (29.3 kg/d) and milk contents (43.3, 31.9 and 47.9 g/kg fat, protein and lactose, respectively). In situ ruminal fractional rate of degradation of starch decreased linearly from 0.098 to 0.059 /h as maturity increased from MS25 to MS40. Apparent total-tract digestibility of DM, OM, CP, NDF, crude fat, starch and gross energy (GE) reduced linearly with maturity. Treatments did not affect ruminal pH, VFA and ammonia concentrations, and VFA molar proportions. The concentration of C18:3n-3 in milk fat decreased linearly, and the concentration of C18:2n-6 and the n-6:n-3 ratio increased linearly with maturity. A quadratic response occurred for the total saturated fatty acid concentration and total mono-unsaturated fatty acid concentration in milk fat with respectively highest and lowest concentration with T28. Daily CH_4 production (390, 400, 386 and 361 g/d), CH_4 production relative to DMI (21.7, 23.0, 21.0 and 20.1 g/kg) and relative to GE intake (0.063, 0.067, 0.063 and 0.060 MJ/MJ) (for T25, T28, T32 and T40, respectively) decreased linearly with maturity. Also CH_4 emission relative to fat- and protein-corrected milk (FPCM) tended to decrease linearly with maturity (13.0, 13.4, 13.2 and 12.1 g/kg of FPCM, for T25, T28, T32 and T40, respectively). Intake of GE and ME, and energy retained, all expressed per unit of metabolic body weight did not differ among treatments. Nitrogen intake, N use efficiency (milk N/N intake) and N balance were not influenced by dietary treatments. Increasing maturity of whole-plant maize

at harvest may offer an effective strategy to decrease CH_4 losses with feeding WPMS without negatively affecting animal performance.

Key words: maturity; maize silage; starch; methane; dairy cow

INTRODUCTION

Whole-plant maize silage (WPMS) is a major forage component in dairy cattle rations in many parts of the world, with a high energy content and generally good ensiling characteristics (Khan et al., 2015). The nutritive value of WPMS is highly dependent on its digestibility and content of starch. Starch content of WPMS is mainly affected by stage of maturity of the plant at harvest (Johnson et al., 1999). The advancing maturity of the maize crop during the grain-filling period increases the content of DM and starch, and decreases the content of NDF (Tolera et al., 1998; Johnson et al., 1999; Phipps et al., 2000). In addition to the change in nutritional composition of the maize crop, the vitreousness of maize kernels (i.e., the proportion of vitreous in the total endosperm) increases with maturity (Correa et al., 2002; Ettle et al., 2001). This increase in vitreousness with maturity is associated with reduced ruminal starch degradation of maize (Philippeau and Michalet-Doreau, 1997; Ettle et al., 2001) and an increased postruminal starch digestion (Sutton et al., 2000). A reduced ruminal starch rate of degradation may lead to an improved animal performance as there is no associated loss of energy with methane (CH_4) production (Dijkstra et al., 2011), although an advanced maize maturity leading to DM contents beyond 35% may reduce milk production due to decreased feed intake and maize silage digestibility (Khan et al, 2015).

The importance of changes in the nutritional components of WPMS with increasing maturity with respect to nutritional and production responses by ruminants livestock are well documented in several studies (Bal et al., 1997; Cammell et al., 2000; Phipps et al., 2000; Johnson et al., 2002a). However, only limited systematic studies have evaluated the effects of harvest maturity of whole-plant maize for maize silage on CH_4 emissions in lactating dairy cows, though increasing the starch content in ruminants' diet is suggested as an effective way of decreasing CH_4 emission intensity (Hristov et al., 2013). Feeding high starch diets to ruminants lowers rumen pH and generally favors propionate production at the expense of acetate (Bannink et al., 2008; Lettat et al., 2013) and may decrease the population of protozoa (Dijkstra et al., 1994; Martin et al., 2010; Morgavi et al., 2010). These effects reduce the availability of hydrogen to be utilized by rumen methanogens to produce CH_4.

In beef cattle fed diets containing on average 77% maize silage, increasing WPMS maturity at harvest tended to reduce CH_4 relative to feed DM and carcass gain, where CH_4 emission was measured using the sulfur hexafluoride (SF_6) method and maize silage was offered ad libitum (Mc Geough et al., 2010). In contrast, Cammell et al. (2000) did not find significant differences in CH_4 emission, ME intake, and in the partitioning of ME to heat or

milk in dairy cattle fed diets based on maize silage made from whole-plant maize harvested at different stages of maturity and measured using respiration chambers. The proportion of WPMS in the diet was limited to 40% of the total diet DM, and the authors suggested that observed effects of maturity of maize silage at harvest would be greater with a higher inclusion of maize silage. In view of these findings and of the observed effect of maturity of maize at harvest, it is important to further evaluate the potential of advanced maturity of maize as a CH_4 mitigation strategy when including a high proportion of WPMS in diets of lactating dairy cows.

The primary objective of this study was to evaluate the effects of increasing maturity of whole-plant maize at harvest on CH_4 emission by lactating dairy cows consuming WPMS based diets. We hypothesized that advancing whole-plant maize maturity at harvest increases starch content, and decreases NDF content and ruminal fractional degradation rate of starch, and thus enhance postruminal starch supply or may cause a shift in the pattern of rumen VFA production towards more propionate. These changes are expected to decrease CH_4 yield (CH_4 per unit of feed intake) and CH_4 emission intensity (CH_4 per unit of milk produced).

MATERIALS AND METHODS

Silage preparation

The WPMS was prepared from a single maize variety (LG30218; Limagrain, Rilland, the Netherlands), which is a commonly used maize variety by farmers in the Netherlands. Maize was planted on sandy soil at a density of 100,000 seeds per ha and row spacing of 0.78 m at the experimental farm of Wageningen University (Achterberg, the Netherlands) in mid-May 2013. The maize field was fertilized with cattle slurry (40 tons/ha) containing 3.8 kg of nitrogen (N), 1.5 kg of P_2O_5 and 4.8 kg of K_2O per ton; triple superphosphate (75 kg/ha) and calcium ammonium nitrate (125 kg/ha). The whole-plant maize was harvested and ensiled at a very early (25.1% DM, harvested on September 20; MS25), early (27.7% DM, harvested on September 28; MS28), medium (32.0% DM, harvested on October 9; MS32) and late (40.3% DM, harvested on October 31; MS40) stage of maturity. To determine the stage of maturity at harvest, five maize plants were randomly selected throughout the field once weekly, harvested, chopped and dried at 60°C in a forced-air oven at least for 48 h. Once the DM content aimed was attained, the whole maize plants were harvested with a precision chop harvester (Claas Jaguar 980 with a 6-row maize head) with chop length and cutting height set

to 7 mm and 120 mm, respectively, and kernel processer set to 1 mm to ensure that all kernels were sufficiently crushed or processed.

Immediately after harvest, tractor-trailers with the harvested plant materials were weighed and unloaded into one-side walled concrete short silos (13.8 m long, 2.9 m wide and 0.6 m high) for MS25, MS28 and MS32, and a long silo (40.0 m long, 2.5 m wide and 0.8 m high) for MS40. A long silo was used to be able keep the package density similar among different harvest maturity since compaction is more difficult when DM content is higher. The silage heaps were mechanically compacted with a heavy weight tractor and a loader. After the silos were filled and compacted, the sides and top of the silage heap was covered with two layers of 0.15 mm black polyethylene plastic sheet. To minimize the damage to the polyethylene sheet, the plastic sheet was further covered with a knitted protection sheet, and sand was placed on the top of the sheet to ensure effective air tight sealing. Ensiling was done without addition of inoculum and completed within approximately 3 h of harvest at the field.

To evaluate the fermentation characteristics and DM content of the silages prior to the animal feeding experiment, samples using a hollow drill were taken on d 110, 102, 91 and 69 after ensiling for MS25, MS28, MS32 and MS40, respectively.

Experimental design, feeds and feeding

The experiment was conducted at the Animal Research Facilities of Wageningen University, Wageningen, The Netherlands, during April to May 2014. All experimental procedures were approved by the institutional Animal Care and Use Committee of Wageningen University (Wageningen, The Netherlands) and carried out under the Dutch law on animal experimentation.

Twenty-eight Holstein-Friesian dairy cows (8 primiparous and 20 multiparous) of which eight fitted with permanent rumen cannula (10 cm i.d., Type 1C, Bar Diamond Inc., Parma, ID) were allocated to seven blocks of four cows each, based on parity, DIM (103 ± 18.4 d; mean ± SD) and fat- and protein-corrected milk (FPCM; 38.0 ± 9.0 kg/d) at the start of the trial, and presence of a rumen cannula. Cows within blocks were randomly assigned to one of four experimental treatments that consisted of (DM basis) 75% WPMS, 20% concentrate and 5% wheat straw. Treatments were designated as T25, T28, T32 and T40, respectively, to reflect the DM contents of whole-plant maize at harvest. Concentrate and wheat straw were similar for all treatments. Concentrate was produced at Research Diet Services B.V. (RDS, Wijk Bij Duurstede, The Netherlands) in one batch and hence expected to be of uniform composition throughout the experiment. The concentrate was composed of (g/kg of DM)

soybean meal (47% CP) = 327, formaldehyde treated soybean meal (RUMI–S) = 275, maize = 255, rapeseed meal = 52, limestone = 31, urea = 20, sodium chloride = 15, mono-calcium phosphate = 12, magnesium oxide = 11, chromium oxide = 1.5 and vitamin-mineral premix = 2.0. The analysed chemical composition of the concentrate was ash = 109, CP = 403, NDF = 127, ADF = 67, ADL= 7, crude fat = 27 and starch = 183 (all in g/kg of DM), and GE = 17.8 MJ/kg of DM. Similarly, the wheat straw consisted of ash = 107, CP = 31, NDF = 758, ADF = 475, ADL = 61, crude fat = 11 and starch = 10 (all in g/kg of DM), and GE = 17.6 MJ/kg of DM. The chemical composition of the dietary treatments calculated from the chemical analyses and proportion of each diet ingredient is presented in Table 1.

Whole-plant maize silage and wheat straw mixtures were prepared twice weekly using a self-propelled mixer wagon (Strautmann Verti-Mix 500, Bad Laer, Germany) equipped with a cutter loader system and an electronic weighing unit and stored in a cooling unit (6°C) prior to feeding. The daily rations were offered individually in two equal meals at 0600 h and 1600 h, with any feed refusal being removed before feeding and recorded. The roughage portion and concentrate (which was in the form of meal) were thoroughly mixed manually when fed.

Five days before the start of the adaptation period cows from the same block were housed in the free-stall barn in a group to acclimatize gradually to an increased level of inclusion of non-experimental maize silage and a decreased level of concentrate in the diet (compared with the regular dairy ration they received previously), which was considered a pre-adaptation period. After this period, dietary treatments were introduced and cows were individually housed in tie-stalls for a 12 d adaptation period. During the first 8 d of the adaptation period cows were fed ad libitum. From d 9 onwards feed intake was restricted per block, to 95% of the ad libitum feed intake of the animal with the smallest DMI during the five preceding days, as described previously by Van Zijderveld et al. (2011) but at all times a minimum DMI of at least 80% of the ad libitum intake of cows with the greatest DMI within block was ensured. Diet allowance was restricted to reduce variation in feed intake between animals per block and to ensure complete consumption of the ration during the measuring days.

Following the adaptation period, cows were individually housed in one of four identical climate-controlled respiration chambers (CRC) for CH_4 measurements and measurements of energy and N balance. Cow-treatment combinations were randomly assigned to one of the four CRC. Cows entered the chambers on d 13 at 1500 h and left the chambers on d 17 at 0900 h. Detailed information on the technical aspects of the chambers design, calibrations and gas analysis has been reported by Van Gastelen et al. (2015). Briefly, in each CRC (volume

Table 1. Chemical composition of maize silages and diets, and fermentation profile of maize silages differing in maturity at harvest.

Item	Maize silage[2]				Treatment[3]			
	MS25	MS28	MS32	MS40	T25	T28	T32	T40
Growing days[1]	128	136	147	169	–[6]	–	–	–
DM content (g/kg)	283	292	318	396	437	444	463	522
Chemical composition[4]								
Ash	39	37	37	35	56	55	55	53
CP	83	83	80	79	145	145	142	142
NDF	407	394	359	349	369	359	332	325
ADF	242	233	207	195	219	212	193	183
ADL	11	11	9	10	13	12	11	12
Crude fat	26	27	25	24	26	26	25	24
Starch	275	305	356	385	243	266	304	326
GE (MJ/kg of DM)	18.9	18.8	18.7	18.6	18.6	18.5	18.4	18.4
Fermentation parameters								
pH	3.7	3.7	3.8	3.8	–	–	–	–
Lactic acid[4]	18.0	17.0	19.0	20.6	–	–	–	–
Butyric acid[5]	ND	ND	ND	ND	–	–	–	–
Propionic acid[5]	ND	ND	ND	ND	–	–	–	–
Ethanol[4]	0.4	2.8	1.4	0.1	–	–	–	–
N-NH_3 (% of total N)	7.5	7.6	9.1	9.1	–	–	–	–

[1]Number of days from planting until harvesting of the whole-plant for ensiling.
[2]Whole-plant maize was harvested at a DM content of 25, 28, 32 and 40% for MS25, MS28, MS32 and MS40, respectively.
[3]Treatments had roughage – to – concentrate ratio of 80:20 (DM basis). Roughage consisted of (DM basis) 75% maize silage and 5% wheat straw. T25, T28, T32 and T40 contained MS25, MS28, MS32 and MS40, respectively.
[4]Values in g/kg of DM, unless stated otherwise.
[5]Both butyric and propionic acids were not detected in the maize silage.
[6]Not applicable.

35 m^3) relative humidity was maintained at 70% and temperature at 16°C. The ventilation rate was 42 m^3/h per compartment and inlet and exhaust air of each compartment was sampled at 10-min intervals. Gas concentrations and ventilation rates were corrected for pressure, temperature and humidity to arrive at standard temperature pressure dew point volumes of

inlet and exhaust air. Cows were exposed to 16 h of light per day and within the CRC, cows were able to see and hear each other to prevent social isolation. Cows in the chamber were tethered in individual stalls with slatted floor fitted for collection of manure (feces and urine mixture) output. Staff entered each CRC twice daily at 0600 and 1600 h for approximately 30 min for milking and feeding. Animals had unrestricted access to drinking water during the entire experimental period.

Sample collection and measurements

Samples of ruminal fluid from rumen cannulated cows were collected on d 10 and 11 of the adaptation period to measure pH, and determine VFA and ammonia concentrations. Rumen fluid samples (approximately 250 ml) were collected at 0 h (pre-morning feeding), and 1, 2, 3, 4, 6, 8 and 10 h post-morning feeding on both days using the method described by Hatew et al. (2015). The pH of the sample was immediately measured using a portable pH meter (Hanna Instruments Model HI 9024, IJsselstein, The Netherlands). Then, two subsamples were collected. The first sample was acidified with equal volume of 0.85% ortho-phosphoric acid containing 19.68 mM isocaproic acid as internal standard, and stored at −20^{o}C until analysis for VFA by using a gas chromatography (Fisons HRGC MEGA2, Milan, Italy), whereas the second sample was mixed with an equal volume of 10% trichloroacetic acid and stored at −20^{o}C pending analysis for ammonia.

For DM content determination of the feeds, samples from WPMS were collected upon feed preparation and dried at 60^{o}C in a forced-air oven at least for 48 h. Samples of concentrate and wheat straw were collected once weekly and dried at 60^{o}C in a forced-air oven. For the purpose of other chemical analysis, samples from each WPMS offered in the CRC were collected each time the feeds were prepared and stored at −20°C. These samples were later thawed and two subsamples were taken. The first subsample was used for in situ incubation, whereas the second once was dried in a forced-air oven at 60^{o}C waiting for chemical analysis. Orts in the CRC (when present) were removed, weighed and stored at 4°C. At the end of each measurement period, the orts were pooled per cow and a subsample was taken and stored at −20°C pending analysis.

To determine the total-tract apparent digestibility of DM, OM, N, crude fat, starch, NDF and GE, cows were offered chromium oxide incorporated in the concentrate (1.5 g per kg of concentrate DM), which was used as an ingestible marker. Grab fecal samples were collected daily in CRC during milking for 3 consecutive days in addition to the evening of d 13 and morning of d 17. Samples were pooled for individual cows and stored at −20^{o}C. At the end of

the experiment sample for each cow was weighed and mixed before a subsample was taken pending analysis.

Gaseous exchange measurements (O_2 consumption and CO_2 production) were initiated after the cows entered the CRC and recorded for each measurement period and each individual cow following the detailed procedures described by Van Gastelen et al. (2015). Respiration gases and condensed water from the CRC were collected and stored at 4°C pending analysis. For the complete energy and N balance estimation, stainless steel gutter pans were fixed in the gutter behind each cow. At the end of each measurement period in CRC (i.e. d 17 after 0900 h), manure (feces and urine mixture) output of each cow was quantitatively collected and transferred into a large container and total weight was recorded. After thorough mixing, two samples (ca. 500 ml) were taken and stored at −20°C pending analysis for energy and N contents. Determination of the amount and energy content of the manure and direct measurement of CH_4 emission allowed estimating the ME intake, and simultaneous measurement of gaseous exchange allowed to indirectly estimate heat production according to the relationship previously described (Brouwer, 1965). Nitrogen lost in the form of ammonia that may result from mixing of feces and urine was captured and quantified as described by Hatew et al. (2015).

Feed intake, milk production and CH_4 emission parameters recorded during the last three days of each period were used for statistical analyses. For calculation of energy and N balance, average DMI per cow was computed for d 13 (0800 h of d 13) to d 16 (0800 h of d 17) of each period, based on daily records of total diet offered and refusals for each cow. Cows were weighed immediately after entering and just before leaving the CRC. The gas measurements during opening of the CRC for milking and feeding purpose were excluded from data analyses.

Milk samples from a.m. and p.m. milking were collected for a total of eight milkings (starting from d 13 afternoon to d 17 morning) of each period and analysed for fat, protein, lactose and urea. Average daily concentrations of milk components were calculated after adjusting for milk yield of each milking. For energy and N balance determination, milk yield in CRC was recorded and a milk sample at each milking (5 g/kg of milk yield) was collected, pooled per cow and stored at −20^{o}C pending analyses for energy and N contents. In addition, representative sample (5 g/kg milk yield) for each cow was obtained for milk fatty acids (FA) composition determination according to the method described by Van Gastelen et al. (2015).

Estimation of in situ ruminal degradability

In situ ruminal degradation characteristics of the WPMS were determined in a separate experiment using three lactating dairy cows fitted with ruminal cannula. Cows were fed ad libitum a mixed diet composed of (DM basis) 39% WPMS and 61% grass silage and a commercial concentrate according to milk production up to a maximum of 7 kg/d. The cows were on average 217 ± 0.6 (mean ± SD) DIM and producing 27.4 ± 1.7 kg/d of milk. A pooled subsample of each WPMS was obtained from samples collected during the CH_4 measurement periods in CRC (d 13 to 17) and stored frozen (−20°C). Frozen samples of each WPMS were cut using a food cutter (FEUMA FGC 10-2, Gößnitz, Germany) for 1 min. Then, approximately 5.0 g (DM basis) of each silage was weighed into four nylon bags per incubation time for each cow. Bags were incubated in the rumen of each of the three cows for 3, 6, 12, 24, 48, 96 and 336 h using the all–in all–out procedure. Incubations started at the same time (0800 h) for all time points and incubations were completed within three weeks. Upon removal of bags from the rumen, bags were washed using a modified rising method (de Jonge et al., 2013). Briefly, two nylon bags were placed in a glass vessel (Ø 19 cm, 7 cm height) containing 500 mL buffer solution (12.2 g/L $NaH_2PO_4 \cdot H_2O$ and 8.9 g/L $Na_2B_4O_7 \cdot 10H_2O$, adjusted to pH 6.2 with hydrochloric acid). The vessels were placed in a mechanical shaker (Julabo SW-20c; Julabo GmhB Seelbach, Germany) and were shaken during 60 min at 40 spm (strokes per minute) at room temperature. Rinsed nylon bags were dried at 60°C in a forced-air oven and weighed. Dried residues from four bags for each cow, treatment and incubation time were pooled to one sample, ground and analyzed for DM, ash, starch and NDF contents.

Data of in situ ruminal disappearance of starch, OM and NDF were used to determine rumen degradation characteristics of starch, OM and NDF of each maize silage. An exponential first-order model: proportion of residue present at time $t = U + D \times e^{(-kd \times t)}$ was used, with the nonlinear procedure of SAS (SAS Institute Inc., 2010) to estimate the parameter values, with potentially degradable fraction (D), undegradable fraction (U) and fractional rate of degradation of the D fraction (k_d) constrained to be positive. The washout fraction (W) which is assumed to be rapidly degradable was calculated as 1 – D – U. It is assumed that the U and W fractions were zero for starch and NDF, respectively, and no lag time was included in the model for NDF degradation. Effective rumen degradability (ERD) was calculated as described by Ørskov and McDonald (1979) assuming a fractional rumen outflow rate of 0.045, 0.060 and 0.020 /h for OM, starch and NDF, respectively.

Sample preparation and analytical procedures

Prior to analyses, samples were prepared as described by Hatew et al. (2015) and dried at 60^{o}C in a forced-air oven. All analyses including determination of ruminal VFA concentration and milk composition were carried out according to the methods previously reported by Hatew et al. (2015), except for NDF, ADF and ADL which were analysed using an ANKOM^{2000} fibre analyser (Ankom Technology, Macedon, NY, USA).

The fermentation characteristics of WPMS were measured on aliquots obtained with maceration. Briefly, 30 g of a WPMS sample was weighed into a stomacher bag, diluted with 270 ml distilled water and mixed vigorously for 5 min. The pH of the aliquots was measured immediately using a bench top pH meter (Hanna Instruments pH 300 GLP, Amorim Póvoa de Varzim, Portugal). About 30 ml of the aliquot sample was centrifuged at 25,000 × g for 10 min, and two samples from the supernatants were collected. Each sample was acidified with equal volume of 10% trichloroacetic acid or with 0.85% ortho-phosphoric acid for ammonia and VFA analyses, respectively, and stored at −20^{o}C. VFA concentration was analysed using gas chromatography (Fisons HRGC MEGA2, Milan, Italy), and ammonia concentration was determined by a colorimetric method (Scheiner, 1976). Lactic acid in the supernatant was analysed by HPLC and ethanol determined enzymatically according to the Boehringer-Mannheim method (1989).

Milk FA composition was analysed through gas chromatography as described in detail by Van Gastelen et al. (2015). Results of FA were expressed as grams per 100 g of total FA.

Statistical analysis

All measurements from three cows in CRC were excluded from the statistical analyses. One cow (receiving diet T32) was removed due to mastitis and two cows (receiving diet T40) because of large feed refusals in combination with an irregular pattern of feed intake and milk yield. Prior to analyses, data for intake, digestibility, milk parameters, CH_4 emission parameters, energy and N balance were averaged per period and cow. Data were checked for normality using the UNIVARIATE procedure of SAS, and data were transformed when appropriate using a natural logarithm function and exponential function to back-transform the least square means and standard error of the means.

All data on feed intake, milk yield and composition, apparent total-tract digestibility, CH_4 parameters, energy and N balance were analysed as a completely randomized block design using the MIXED procedure in SAS (SAS Institute Inc., 2010). The model included the fixed effect of treatment (DM content of WPMS at harvest) and a random effect of block. Each

block of four animals was completed within one experimental period; thus, period effect was completely confounded with block effect. Similarly, data on in situ rumen degradation of the maize silages were analysed using the MIXED procedure in SAS with treatment as fixed effect and cow as random effect. The Kenward-Roger method was used to calculate the denominator degree of freedom. Autoregressive 1 (AR 1), variance component (VC), compound symmetry (CS) and unstructured (UN) covariance structures were tested for each analyses and the covariance structure with the lowest overall Akaike's information criterion values (i.e., variance component) was selected.

Data for ruminal pH, ammonia and VFA concentrations were averaged per time point per cow and subjected to repeated-measures ANOVA to take repeated samples within the same animal into account. This model included cow and block as random effects, and treatment, time of sampling and the interaction of treatment and time of sampling as fixed effects. Because of the inherent unbalanced sampling-time interval, spatial power (POW) variance components were used as the covariance structure of choice to account for within-cow variation.

Orthogonal polynomial contrasts (linear and quadratic) were used to examine the effects of treatment (DM content of whole-plant at harvest) on response variables. Due to unequally spaced treatments, polynomial coefficients were generated by using the orthogonal polynomial (ORPOL) function in IML (interactive matrix language) procedure of SAS.

All results are reported as least square means, and significance of linear or quadratic treatment effects was declared at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$.

RESULTS

Chemical composition of silages and diets

As whole-plant maize maturity at harvest progressed, WPMS starch content increased from 275 to 385 g/kg of DM (MS25 vs. MS40) coinciding with a reduction of NDF content (from 407 to 349 g/kg of DM) and ADF content (from 242 to 195 g/kg of DM) (Table 1). The ADL, CP, crude fat and GE contents of WPMS varied within a rather narrow range only, and volatile fermentation products in WPMS were minor. The same pattern of change for starch, NDF and ADF contents occurred for dietary treatments. The silages were of good quality as reflected by low pH, small concentration of N-NH_3 and no detectable presence of butyric and propionic acids. There was virtually no visible discoloration or surface spoilage in the silos.

In situ ruminal degradation of maize silages

The ruminal fractional rate of degradation of starch (P = 0.006) and NDF (P = 0.040) of the WPMS linearly decreased with increased harvest maturity of whole-plant maize (Table 2). For OM, the D-fraction tended to increase quadratically, and the k_d tended (P = 0.092) to decrease quadratically with maturity. The estimated effective rumen degradability (ERD) of

Table 2. In situ rumen degradation characteristics of maize silages differing in maturity at harvest.

Degradation parameter[1]	Maize silage[2]				SEM	*P*-value	
	MS25	MS28	MS32	MS40		Linear	Quadratic
Starch							
W	0.27	0.27	0.29	0.26	0.026	0.530	0.174
D	0.73	0.73	0.71	0.74	0.026	0.530	0.174
k_d	0.098	0.082	0.074	0.059	0.0155	0.006	0.401
ERD	0.71	0.68	0.67	0.62	0.038	<0.001	0.862
Organic matter							
W	0.21	0.19	0.15	0.19	0.012	0.008	<0.001
U	0.30	0.30	0.29	0.28	0.023	0.024	0.715
D	0.49	0.52	0.56	0.54	0.033	0.003	0.001
k_d	0.026	0.026	0.026	0.022	0.0027	0.006	0.092
ERD	0.39	0.38	0.35	0.36	0.008	0.002	0.001
NDF							
U	0.46	0.48	0.50	0.46	0.042	0.993	0.090
D	0.54	0.52	0.50	0.54	0.042	0.993	0.090
k_d	0.017	0.013	0.012	0.012	0.0015	0.040	0.116
ERD	0.25	0.20	0.19	0.20	0.012	0.016	0.006

[1]W = washable fraction (g/g) and assumed to be zero for NDF; D = potentially degradable fraction (g/g); U = undegradable fraction (g/g) and assumed to be zero for starch; k_d = fractional degradation rate constant of the potentially degradable fraction (/h); ERD = effective rumen degradability (g/g).
[2]Whole-plant maize was harvested at a DM content of 25, 28, 32 and 40% for MS25, MS28, MS32 and MS40, respectively.

starch decreased linearly (P < 0.001), and the ERD of OM (P = 0.016) and NDF (P = 0.001) decreased quadratically with maturity. The ERD of starch, OM and NDF of the WPMS was reduced by 13, 8 and 20%, respectively, with advanced maturity of the maize at harvest.

Ruminal fermentation characteristics of diets

Rumen pH, total VFA concentration, VFA molar proportions and ammonia concentration were not affected by the type of WPMS, except for a tendency for the molar proportion of propionate to decrease quadratically (P = 0.056) and the ratio of acetate to propionate to increase linearly (P = 0.089) with advancing maize harvest maturity (Table 3). All rumen var-

Table 3. Rumen fluid characteristics of lactating dairy cows fed diets based on maize silage differing in maturity at harvest.

	Treatment[1]					*P*-value	
Item	T25	T28	T32	T40	SEM	Linear	Quadratic
Rumen pH	6.49	6.43	6.56	6.44	0.032	0.631	0.197
Total VFA (mM)	102.8	108.6	96.5	105.8	3.37	0.944	0.277
VFA (mol/100 mol)							
Acetate (A)	63.8	65.2	63.9	65.1	0.50	0.291	0.800
Propionate (P)	20.2	18.7	17.9	18.5	0.51	0.096	0.056
Butyrate	12.0	11.8	14.0	11.7	0.92	0.980	0.193
Valerate	1.5	1.5	1.6	1.6	0.04	0.171	0.140
Iso-acids[2]	2.5	2.8	2.7	3.1	0.20	0.106	0.803
A:P	3.2	3.5	3.6	3.6	0.09	0.089	0.169
Ammonia (mg/l)	132.3	137.7	125.7	97.2	13.06	0.131	0.274

[1]Treatments had roughage – to – concentrate ratio of 80:20 (DM basis). Roughage consisted of (DM basis) 75% maize silage and 5% wheat straw. T25, T28, T32 and T40 contained maize silage made from whole-plant maize harvested at a DM content of 25, 28, 32 and 40%, respectively.
[2]Iso-acids = isobutyrate + isovalerate.

iables were affected by time of rumen sampling (P < 0.001; data not shown). In general, rumen pH initially decreased after morning feeding and increased again several hours later, whereas VFA concentration showed the opposite pattern (data not shown).

Animal performance

Intake of DM (DMI), OM, CP and GE were unaffected by WPMS made from whole-plant maize harvested at different stages of maturity (Table 4). Starch intake increased quadratically (P < 0.001), but NDF, ADF and crude fat intake decreased linearly ($P \leq 0.002$) with maturity. Apparent total-tract digestibility of DM (P = 0.002), OM (P = 0.003), CP (P = 0.001), NDF (P < 0.001), crude fat (P < 0.001), starch (P < 0.001) and GE (P = 0.002) decreased linearly with maturity.

Milk yield, fat- and protein-corrected milk production, and milk fat, protein, lactose and urea contents, and milk fat, protein and lactose yield were all unaffected by maturity of maize at harvest (Table 5).

Table 4. Intake and apparent total-tract digestibility of nutrients in lactating dairy cows fed diets based on maize silage differing in maturity at harvest.

	Treatment[1]					*P*-value	
Item	T25	T28	T32	T40	SEM	Linear	Quadratic
Intake (kg/d, unless otherwise stated)							
DM	18.0	17.5	18.5	18.0	0.84	0.478	0.329
OM	17.1	16.5	17.5	17.1	0.80	0.416	0.377
CP	2.61	2.53	2.65	2.57	0.125	0.780	0.570
NDF	6.67	6.27	6.17	5.85	0.294	<0.001	0.121
ADF	3.97	3.69	3.57	3.30	0.173	<0.001	0.064
Crude fat	0.46	0.44	0.46	0.40	0.024	0.002	0.117
Starch	4.38	4.65	5.62	5.88	0.235	<0.001	<0.001
GE (MJ/d)	335.8	323.0	341.2	331.1	15.6	0.931	0.547
Digestibility (%)							
DM	70.7	71.9	69.1	66.9	1.48	0.002	0.546
OM	72.5	73.7	71.2	69.0	1.43	0.003	0.428
CP	70.3	70.0	66.8	66.1	1.31	0.001	0.234
NDF	53.5	55.2	47.4	44.0	2.82	<0.001	0.857
Crude fat	79.2	78.0	75.0	71.7	1.23	<0.001	0.701
Starch	98.8	98.8	98.5	97.8	0.15	<0.001	0.248
GE	70.8	71.7	69.0	66.9	1.46	0.002	0.669

[1]Treatments had roughage – to – concentrate ratio of 80:20 (DM basis). Roughage consisted of (DM basis) 75% maize silage and 5% wheat straw. T25, T28, T32 and T40 contained maize silage made from whole-plant maize harvested at a DM content of 25, 28, 32 and 40%, respectively.

Milk fatty acid composition

The concentration of C4:0 in milk fat decreased linearly (P = 0.019) whereas concentration of some medium-chain milk FA (C10:0, C11:0 and C13:0) increased linearly ($P \leq 0.032$) with maturity of maize at harvest (Table 6). Concentration of C16:0 decreased quadratically (P = 0.003), and C16:1 *trans*-9, iso C17:0, C18:1 *cis*-9 and C18:1 *cis*-13 increased quadratically ($P \leq 0.040$). Concentration of 18:2n-6 increased linearly (P = 0049)

and the inverse was observed for C18:3n-3 (P = 0.002). The concentration of C18:3n-6 and C20:5n-3 decreased quadratically ($P \leq 0.037$). Total saturated FA decreased (P = 0.030) and monounsaturated increased (P = 0.034) quadratically, and the ratio of n-6:n-3 increased linearly ($P < 0.001$) with maturity.

Table 5. Milk yield and composition of dairy cows fed diets based on maize silage differing in maturity at harvest.

	Treatment[1]					*P*-value	
Milk parameter	T25	T28	T32	T40	SEM	Linear	Quadratic
Yield (kg/d)							
Milk	30.8	28.9	28.6	28.7	0.48	0.267	0.278
FPCM[2]	30.2	30.0	29.7	30.1	1.57	0.962	0.614
Fat	1.22	1.27	1.24	1.29	0.069	0.325	0.994
Protein	0.95	0.91	0.94	0.91	0.049	0.392	0.823
Lactose	1.46	1.41	1.37	1.35	0.080	0.171	0.446
Composition[3]							
Fat	40.1	44.3	43.3	45.4	2.00	0.110	0.500
Protein	31.1	31.5	32.7	32.3	0.88	0.313	0.375
Lactose	47.7	48.7	47.8	47.2	0.47	0.160	0.223
Urea (mg/dl)	20.1	20.2	17.5	18.1	1.56	0.196	0.403

[1]Treatments had roughage – to – concentrate ratio of 80:20 (DM basis). Roughage consisted of (DM basis) 75% maize silage and 5% wheat straw. T25, T28, T32 and T40 contained maize silage made from whole-plant maize harvested at a DM content of 25, 28, 32 and 40%, respectively.
[2]Fat- and protein-corrected milk = [0.337 + 0.0116 × fat (g/kg) + 0.0060 × protein (g/kg milk)] × milk yield (kg/d).
[3]Values are in g/kg milk, unless stated otherwise.

Methane production, and energy and N balance

Daily CH_4 output (P = 0.020) and CH_4 production relative to DMI ($P < 0.007$) decreased linearly, and CH_4 energy loss as a fraction of GE intake tended to decrease quadratically (P = 0.051) with increasing maturity of WPMS at harvest (Table 7). Methane emission intensity (CH_4 per unit of FPCM) tended to decrease linearly (P = 0.058) with maturity and was lower for T40 (mean = 12.1 g/kg of FPCM) compared with other treatments (mean = 13.2 g/kg of FPCM). Similarly, CH_4 produced as a proportion of OM digested tended to decrease linearly (P = 0.054) with maturity.

Table 6. Milk fatty acid composition of lactating dairy cows fed diets based on maize silage differing in maturity at harvest.

	Treatment[1]					*P*-value	
FA (g/100 g of FA)	T25	T28	T32	T40	SEM	Linear	Quadratic
C4:0	3.72	3.76	3.51	3.44	0.091	0.019	0.696
C6:0	2.26	2.28	2.21	2.31	0.059	0.609	0.450
C8:0	1.18	1.18	1.16	1.29	0.047	0.085	0.273
C10:0	2.58	2.64	2.57	3.01	0.131	0.032	0.252
C11:0	0.03	0.02	0.04	0.06	0.007	0.009	0.744
C12:0	2.97	3.01	2.95	3.40	0.163	0.075	0.332
C13:0	0.08	0.08	0.09	0.11	0.008	0.016	0.632
C14:0	11.10	11.47	10.99	11.35	0.300	0.799	0.773
iso C14:0	0.09	0.08	0.09	0.08	0.006	0.088	0.971
C14:1 *cis*-9	1.03	0.88	1.02	0.95	0.083	0.784	0.842
C15:0	0.91	0.86	0.98	0.98	0.047	0.158	0.790
iso C15:0	0.23	0.21	0.20	0.20	0.010	0.146	0.248
anteiso C15:0	0.44	0.42	0.42	0.44	0.021	0.840	0.490
C16:0	31.79	35.21	34.79	30.81	0.978	0.141	0.003
iso C16:0	0.19	0.20	0.21	0.20	0.017	0.601	0.560
C16:1 *cis*-9	1.60	1.49	1.56	1.69	0.104	0.330	0.378
C16:1 *trans*-9	0.20	0.18	0.18	0.21	0.008	0.452	0.029
C17:0	0.60	0.55	0.59	0.58	0.024	0.996	0.624
iso C17:0	0.39	0.37	0.34	0.39	0.018	0.746	0.014
anteiso C17:0	0.43	0.44	0.43	0.48	0.020	0.116	0.334
C17:1 *cis*-9	0.29	0.24	0.27	0.30	0.020	0.232	0.098
C18:0	9.73	9.36	9.28	9.48	0.443	0.800	0.489
C18:1 *cis*-9[2]	19.48	17.17	17.91	19.99	0.835	0.283	0.040
C18:1 *cis*-12	0.27	0.29	0.29	0.29	0.018	0.676	0.575
C18:1 *cis*-13	0.15	0.12	0.13	0.16	0.012	0.221	0.022
C18:1 *trans*-6	0.28	0.28	0.26	0.27	0.017	0.792	0.480
C18:1 *trans*-9	0.18	0.18	0.18	0.18	0.010	0.812	0.930
C18:1 *trans*-10	0.31	0.34	0.33	0.39	0.026	0.059	0.739
C18:1 *trans*-11	1.06	0.89	0.99	0.86	0.079	0.194	0.825

Table 6. (continued)

FA (g/100 g of FA)	Treatment[1]				SEM	*P*-value	
	T25	T28	T32	T40		Linear	Quadratic
C18:1 *trans*-15 + C18:1 *cis*-11	0.82	0.74	0.75	0.90	0.051	0.164	0.064
Total CLA[3]	0.45	0.39	0.44	0.37	0.034	0.091	0.724
C18:2n-6	1.59	1.41	1.45	1.72	0.065	0.049	0.060
C18:3n-3	0.28	0.22	0.22	0.20	0.013	0.002	0.115
C18:3n-6	0.12	0.09	0.09	0.07	0.004	<0.001	0.037
C19:0	0.17	0.15	0.15	0.15	0.005	0.171	0.076
C20:0	0.13	0.13	0.13	0.14	0.007	0.071	0.482
C20:1 *cis*-11	0.06	0.05	0.05	0.06	0.006	0.418	0.195
C20:3n-6	0.08	0.08	0.09	0.08	0.006	0.586	0.295
C20:4n-3	0.02	0.01	0.02	0.00	0.005	0.055	0.712
C20:4n-6	0.12	0.11	0.12	0.12	0.007	0.579	0.962
C20:5n-3	0.05	0.04	0.04	0.04	0.002	0.001	0.028
C21:0	0.04	0.04	0.04	0.04	0.002	0.959	0.382
C22:0	0.05	0.05	0.05	0.05	0.004	0.487	0.747
C22:4n-6	0.01	0.01	0.01	0.01	0.005	0.765	0.780
C22:5n-3	0.08	0.07	0.07	0.07	0.004	0.188	0.735
C24:0	0.03	0.03	0.03	0.03	0.004	0.916	0.732
SFA[4]	69.13	72.52	71.27	69.11	1.026	0.414	0.030
MUFA[5]	25.73	22.83	23.90	26.25	0.947	0.271	0.034
PUFA[6]	2.72	2.46	2.55	2.63	0.102	0.980	0.130
n-6:n-3 ratio[7]	4.48	5.01	5.09	6.46	0.204	<0.001	0.355

[1]Treatments had roughage – to – concentrate ratio of 80:20 (DM basis). Roughage consisted of (DM basis) 75% maize silage and 5% wheat straw. T25, T28, T32 and T40 contained maize silage made from whole-plant maize harvested at a DM content of 25, 28, 32 and 40%, respectively.

[2]C18:1 *trans*-12 represents the sum of C18:1 *cis*- 9 and C18:1 *trans*-12, as these two FA could not be separated in the analysis. The portion of C18:1 *trans*-12 is considered to be negligible, as this FA always present in small amounts.

[3]Total CLA consists mainly of C18:2 *cis*-9, *trans*-11.

[4]Sum of saturated FA reported in this table.

[5]Sum of monounsaturated FA reported in this table.

[6]Sum of polyunsaturated FA reported in this table.

[7]Ratio between the sum of C18:2n-6, C18:3n-6, C20:3n-6, C20:4n-6, and C22:4n-6 and the sum of C18:3n-3, C20:4n-3, C20:5n-3, and C22:5n-3.

Table 7. Methane production of lactating dairy cows fed diets based on maize silage differing in maturity at harvest.

	Treatment[1]					*P*-value	
Methane parameters	T25	T28	T32	T40	SEM	Linear	Quadratic
CH_4 (g/d)	390	400	386	361	15.9	0.020	0.292
CH_4 (g/kg of DM intake)	21.7	23.0	21.0	20.1	0.75	0.007	0.451
CH_4 (g/kg of FPCM)	13.0	13.4	13.2	12.1	0.57	0.058	0.166
CH_4 (g/kg of OM digested)	31.7	33.0	30.8	30.2	1.10	0.054	0.770
CH_4 (% of GE intake)	6.3	6.7	6.3	6.0	2.44	0.042	0.051

[1]Treatments had roughage – to – concentrate ratio of 80:20 (DM basis). Roughage consisted of (DM basis) 75% maize silage and 5% wheat straw. T25, T28, T32 and T40 contained maize silage made from whole-plant maize harvested at a DM content of 25, 28, 32 and 40%, respectively.

Gross energy intake (GEI) expressed per kilogram of $BW^{0.75}$ per day tended to increase quadratically ($P = 0.099$), and energy lost as CH_4 expressed per kilogram of $BW^{0.75}$ per day decreased quadratically ($P = 0.017$) with maturity (Table 8). All other parameters related to the energy balance and expressed per kilogram of $BW^{0.75}$ per day (i.e., metabolizable energy intake (MEI), MEI:GEI ratio, heat production, energy output in milk, energy retention (ER) total, ER as protein and ER as fat) were unaffected by harvest maturity of WPMS. Similarly, N intake, N excreted in the manure (feces plus urine mixture), N output in milk and N balance were unaffected by increasing maturity. There was no significant difference in N use efficiency (milk N as a fraction of dietary N intake; mean = 34.3%) between treatments. For all dietary treatments, cows were in positive N balance (mean = 16 g/d).

DISCUSSION

The objective of this study was to evaluate the effects of increasing maturity of whole-plant maize at harvest on CH_4 emission by lactating dairy cows consuming WPMS based diets. We hypothesized that increased maturity decreases CH_4 emission intensity due to an increase in starch content, a decrease in ruminal fractional rate of degradation of starch, and a decrease in fibre content, causing an enhanced postruminal starch supply and a shift in the profile of rumen VFA production towards more propionate and less acetate. Indeed, CH_4 emission was lower with advanced maturity irrespective of the unit of expression used. Linear trends for a change from 25 to 40% DM in whole-plant maize at harvest indicate −9.9, −8.0, −6.6 and −7.1% CH_4 when expressed per kg of DMI, per kg of FPCM, per kg of OM digested and as fraction of GE intake, respectively. This corresponds to −0.148, −0.071, and −0.142 g

Table 8. Energy and N balance of lactating dairy cows fed diets based maize silage differing in maturity at harvest.

	Treatment[1]					*P*-value	
Energy and nitrogen (N) parameters	T25	T28	T32	T40	SEM	Linear	Quadratic
Metabolic BW ($kg^{0.75}$)	124	119	117	122	3.8	0.821	0.063
Energy partitioning (kJ/kg of $BW^{0.75}$ per day, unless stated otherwise)							
Gross energy intake (GEI)	2,770	2,793	2,947	2,785	79.1	0.820	0.099
Metabolizable energy intake (MEI)[2]	1,656	1,705	1,699	1,683	53.4	0.941	0.715
MEI:GEI ratio (%)	59.8	61.1	57.6	60.4	1.15	0.968	0.216
Methane production	176	188	183	165	5.2	0.031	0.017
Heat production	887	920	930	896	19.3	0.842	0.142
Milk energy output	753	791	781	769	26.9	0.893	0.331
Total energy retained[3]	16	-5	-13	18	46.6	0.905	0.581
Energy retained as protein[4]	14	24	27	16	7.5	0.969	0.167
Energy retained as fat	2	-29	-40	2	6.8	0.890	0.395
N balance (g/d, unless stated otherwise)							
N intake	426	417	426	419	17.6	0.684	0.969
N excreted in manure	261	250	255	258	12.4	0.885	0.215
N in milk	149	143	145	142	7.3	0.294	0.762
N retained[5]	11	19	21	13	5.8	0.948	0.192
N use efficiency (%)	34.8	34.4	34.0	33.9	0.78	0.431	0.674

[1]Treatments had roughage – to – concentrate ratio of 80:20 (DM basis). Roughage consisted of (DM basis) 75% maize silage and 5% wheat straw. T25, T28, T32 and T40 contained maize silage made from whole-plant maize harvested at a DM content of 25, 28, 32 and 40%, respectively.

[2]ME intake = GE intake – methane production – energy in manure (feces and urine mixture).

[3]Total energy retained = ME intake – heat production – energy in milk.

[4]Energy retained as protein = protein gain (N × 6.25) × 23.6 kJ/g of protein.

[5]N retained = N intake – N excreted in manure – N in milk – N in condensate collected from heat exchanger – N trapped from the outflowing air. Total N from condensate and outflowing air was 5.2, 5.1, 5.2 and 6.7 g/d for T25, T28, T32 and T40, respectively.

CH_4 per kg of DMI, per kg of FPCM, and per kg of digested OM, respectively, and −0.031 as fraction of GE intake, per percent unit increase of DM content of whole-plant maize at harvest. In agreement with other studies (Johnson et al., 1999; Phipps et al., 2000; Fernandez et al., 2004), harvesting whole-plant maize at a late stage of maturity markedly increased starch content, and decreased NDF and ADF content of WPMS at feeding.

In the present study, maize silages were made from a single maize variety and from one field. The maize silage was supplemented with straw and one type of concentrate with low starch content, and the WPMS comprised the major part of the total diet DM (75%) and supplied almost all the dietary starch. This enabled us to investigate the potential of increased maturity of whole-plant maize at harvest as a CH_4 mitigation strategy without potential confounding effects of DMI, concentrate composition and starch not originating from WPMS. The DM content of WPMS at feeding out from the silos ranged from 283 (MS25) to 396 g/kg (MS40). The difference in the DM contents between MS25 and MS28 at feeding were smaller (283 vs. 292 g/kg; MS25 vs. MS28) than the difference in the DM content at harvest (251 vs. 277 g/kg; MS25 vs. MS28) due to losses of moisture as seepage or effluent squeezed from the silo. Although the differences in DM content between these two silages at feeding were minor, the higher starch content, the lower NDF content and the slower fractional rate of degradation of starch of MS28 compared with MS25 showed it was a more mature plant at harvest, indicating that the aim of the present study to create a range of contrasting WPMS was achieved. Currently, a recommended harvest practice is at DM content between 30 and 35% of the maize plant (Khan et al. 2015). In the Netherlands for example, the average starch content of maize silage is in between 30 and 33% (Bannink et al., 2011). Our results suggest that harvesting whole-plant maize at a higher maturity than current practice may have potential to reduce enteric CH_4 emissions.

Ruminal degradation and VFA concentration

Increased maturity of whole-plant maize at harvest reduced in situ effective ruminal starch degradability by 13%. This agrees with the study of Andrae et al. (2001) who reported a 7 to 24% decrease of in situ disappearance of starch when comparing maize silage from two hybrids with DM content of 26 versus 41%. In agreement with the 7% (M32 vs. MS40) decrease in ruminal starch disappearance, Bal et al. (2000) also found an 8% lower ruminal starch disappearance with higher (42%) compared to lower (30%) DM content. In the present study, the rumen fractional rate of degradation of starch was reduced by 40% with advanced maturity. Ruminal starch degradation is shown to be negatively related to maize kernels

vitreousness (Correa et al., 2002). With increasing vitreous endosperm, the concentration of zein proteins increases, whereas that of glutelin proteins decreases (Phipps et al., 2000). The increase in insoluble zein proteins at the expense of the soluble glutelin proteins limits accessibility of starch granules to ruminal microorganisms. Although we did not measure the vitreousness of endosperm of the maize, the in situ data confirm that effective rumen degradability of starch by rumen microorganisms decreased with increasing maturity at harvest.

The increase in starch intake (34%) with increased maturity did not affect ruminal pH and VFA concentrations. The higher starch intake was anticipated to favour propionate production at the expense of acetate (Lettat et al., 2013) as diets with high starch content are often associated with increased propionate in the rumen (Ellis et al., 2008). However, in the present experiment the molar proportion of propionate tended to decrease, and the acetate-to-propionate ratio tended to increase with maturity, possibly related to the decline in fractional rate of degradation of starch, or increased ruminal fractional passage rate relative to ruminal fractional rate of degradation. This result is largely in agreement with Fernandez et al. (2004) who also did not find differences in ruminal pH, total VFA concentration and VFA molar proportions (except for a tendency in propionate molar proportion) when cows were fed diets composed of 80% (DM basis) WPMS made from whole-plant maize harvested at an early (24% DM) and late (32% DM) stage of maturity. Johnson et al. (2002b) found greater ruminal acetate and lower propionate concentrations 2 and 6 h after feeding in rumen fluid of cows offered maize silage from whole-plant maize harvested at more advanced maturity (48% DM) as compared to 34% DM, and explained the effect from decreased ruminal starch digestion rate with advanced maturity. It is important to note that we tested diets with 80% roughage in DM, including 5% wheat straw, which may have increased buffering capacity and chewing activity (Mertens, 1997), causing a lack of effect on rumen pH. In line with our results, also Van Gastelen et al. (2015) found no effect of inclusion of 80% MS versus 80% grass silage in the diet (DM basis) of lactating cows on molar proportion of propionate and rumen pH.

Total-tract digestibility and animal performance

The decrease in faecal OM digestibility with increased maturity of whole-plant maize at harvest could be due to a slight decrease in starch digestibility and a large decline in NDF digestibility, which matches the decrease in digestibility reported for the stover (leaves and stem) portion of the maize plant (Johnson et al., 1999; Tolera et al., 1998). A decrease in faecal starch digestibility only became apparent with WPMS made from the most mature

maize, which agrees qualitatively with the 98.2, 98.1, 98.2 and 96.6% apparent starch digestion observed by Cammell et al. (2000) for a similar range of DM content of maize silage at harvest (23, 28, 33 and 38%, respectively), as used in the present study. Jensen et al. (2005) also recorded a slightly decreased starch digestibility from 100 to 98% with an increase of DM content at harvest from 26 to 40%. Also consistent with our result, the same authors found a significant reduction in faecal NDF digestibility with advanced maturity (from 56 to 43% vs. 54 to 44% in the present study). The increase in starch intake and expected decrease in rumen pH not likely contributed to the lower NDF digestibility observed in the present study, because rumen pH did not differ between treatments. Moreover, the in situ data also indicate reduced fractional rate of degradation of fiber with advanced maturity. The decrease in digestibility of NDF with increasing maturity is presumably the result of an increased lignification, which interferes with enzymatic access to cell wall polysaccharides (Russell et al., 1992). Cone et al. (1993) and Boon et al. (2005) also indicated that increased lignin content, cross-linkage of lignin with fiber and secondary cell wall thickness with advanced maturity explains most of the variation in NDF digestibility of maize silages.

In a recent review, Khan et al. (2015) reported that a low starch:NDF ratio and high moisture content at a very early stage of maturity (25% DM) results in low DMI. This was not the case in the present study and there was no significant difference between treatments since cows were fed restricted. Although in the present study the apparent total-tract digestibility of OM, NDF, CP and starch decreased with increasing maturity, dietary treatments had no effect on milk yield and milk composition. In line with this, Fernandez et al. (2004) also did not find difference in milk yield when cows were fed diets composed of 80% WPMS (DM basis) made from maize plant harvested at an early (24% DM) and late (32% DM) stage of maturity. In contrast, reduced milk yields have been reported for DM contents higher than 35% (Khan et al., 2015), whereas Bal et al. (1997) found an increase in milk yield when cows were fed WPMS with 35% DM compared to 30% DM. The discrepancy between studies might be due to difference in mechanical processing (Andrae et al., 2001; Johnson et al., 1999), the variety of the maize investigated (Ettle et al., 2001; Johnson et al., 2002a), or differences in effects of DM content on rumen fermentation, or on OM, NDF and starch digestibility. For instance, in the study of Bal et al. (1997) starch digestibility was 94.1 and 92.2% for maize silage made from plant harvested at 30 and 35% DM, respectively, whereas in the present study starch digestibility was the same for T28 and T32 (98.8%) and only slightly different for T32 and T40 (98.5 vs. 97.8%).

The FA content of MS was expected to be similar to that reported by Khan et al. (2011), and these affected the milk FA profile. In line with Khan et al. (2015), increased maturity of whole-plant maize at harvest from 25 to 40% DM decreased and increased the concentrations of C18:3n-3 and C18:2n-6 in milk fat, respectively, and increased the ratio of n-6:n-3. The change in these polyunsaturated FA in the milk matches with the change in FA composition of maize plant to be expected with an increase of maturity. The C18:3n-3 is the major FA of the stover part of maize plants, whereas C18:2n-6 is the major FA of the grains or cobs (Khan et al., 2011).

Methane emission and energy and N balance

Despite the absence of differences in DMI between treatments, daily CH_4 production (g/d), CH_4 emission per unit of DMI and per unit of FPCM, and CH_4 energy loss as a fraction of GE intake decreased by 8.6, 9.9, 8.0 and 7.1%, respectively, with a linear increase in the stage of maturity at harvest. A decreased ruminal fractional degradation rate of starch (−40%) and increased starch intake (+34%) with advanced maturity at harvest might have increased duodenal flow of starch (Jensen et al., 2005; Fernandez et al., 2004; Sutton et al., 2000) and hence reduced CH_4 emission intensity. In addition, the marked increase in starch intake may have decreased protozoa, fibrolytic bacteria and methanogenesis (Lettat et al., 2013). In that study using a cDNA-based quantitative PCR method, the authors reported that feeding dairy cows a 60% maize silage diet as compared with a control diet (without maize silage) reduced the richness and the diversity of the bacterial community and the protozoa number. The reduction in CH_4 production was suggested to be related to the decrease in protozoa number. Using a mathematical model, Dijkstra (1994) also showed an initial increase in protozoal biomass in response to an increase in dietary starch content, but a subsequent decline again with further increase in dietary starch content as realized in the present study for the most mature WPMS at harvest.

Methane emission (irrespective of the unit of expression used) was lower with T25 compared to T28, although ruminal fractional rate of degradation of starch and NDF were higher with T25. This might be due to the higher FA content expected in MS25. With the progressive maturity of maize plants the content of C18:3n-3 in stover decreases, related to the decrease in leaf:stem ratio, and to maturation and senescence of the leaves during the grain-filling period (Khan et al., 2011). In addition, C18:3n-3 was clearly present at a higher concentration in milk fat of T25 compared to other treatments. C18:3n-3 in the milk can only originate from the diet (Khan et al., 2011). As reported in a meta-analyses of Patra (2013), the

C18:3n-3 was shown to be associated with inhibition of CH_4 emission to a much larger extent than C18:2n-6, the latter actually increasing in ears of maize with advanced maturity up to some 60 d after flowering (Khan et al., 2011), and this might be the reason for lower CH_4 emission for T25 compared with T28.

In line with previous studies (Cammell et al., 2000; Phipps et al., 2000), an increased maturity had no effects on efficiency of energy utilization for milk production. Similarly, N use efficiency was not affected by dietary treatments. Starch can have a stimulating effect on rumen synthesis (Oba and Allen, 2003) and outflow of microbial protein (Oba and Allen, 2003; Fernandez et al., 2004). No effect was observed on milk protein yield in the present study however. A high N utilization of 34% was achieved, related to the moderate protein content of the diet (144 g CP/kg DM). If dietary CP would not have been sufficient, it may have prevented a further benefit of feeding silage with high starch content on milk yield and N utilization.

CONCLUSIONS

Increasing maturity of maize at harvest markedly increased starch content and decreased NDF content in WPMS, as well as decreased in situ ruminal fractional rate of degradation of starch, which resulted in a reduced CH_4 emission irrespective of the unit of expression used (per kg of DMI, per kg of digested OM, per kg of FPCM, or as a fraction of GE intake). Rumen VFA production profile was not changed, however, and seems unrelated to the observed differences in CH_4. Results of the present study suggest that increasing maturity of whole-plant maize at harvest may offer an effective strategy to decrease CH_4 production of dairy cows with feeding WPMS without negatively affecting animal performance.

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CHAPTER 5

Effects of nitrogen fertilisation rate and maturity of grass silage on methane emission by lactating dairy cows

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ABSTRACT

Grass silage is typically fed to dairy cows in temperate regions. However, in vivo information on methane (CH_4) emission from grass silage of varying quality is limited. We evaluated the effect of 2 rates of N fertilisation of grassland (low fertilisation (LF), 65 kg of N/ha; and high fertilisation (HF), 150 kg of N/ha) and of 3 stages of maturity of grass at cutting: early maturity (EM; 28 days of regrowth), mid maturity (MM; 41 days of regrowth), and late maturity (LM; 62 days of regrowth) on CH_4 production by lactating dairy cows. In a randomised block design, 54 lactating Holstein-Friesian dairy cows (168 ± 11 days in milk; mean ± standard error of mean) received grass silage (mainly ryegrass) and compound feed at 80:20 on dry matter basis. Cows were adapted to the diet for 12 days and CH_4 production was measured in climate respiration chambers for 5 days. Dry matter intake (DMI; 14.9 ± 0.56 kg/day) decreased with N fertilisation and grass maturity. Production of fat- and protein corrected milk (FPCM; 24.0 ± 1.57 kg/day) decreased with grass maturity but was not affected by N fertilisation. Apparent total-tract feed digestibility decreased with grass maturity but was unaffected by N fertilisation except for an increase and decrease in N and fat digestibility with N fertilisation, respectively. Daily CH_4 production per cow (347 ± 13.6 g) decreased with N fertilisation by 4% and with grass maturity by 6%. The smaller CH_4 production with increasing grass maturity was offset by a smaller FPCM and lower feed digestibility. As a result, CH_4 emission intensity increased by 31% per kg of FPCM (15.0 ± 1.00 g CH_4) and by 15% per kg of digestible organic matter intake (33.1 ± 0.78 g CH_4) with increasing grass maturity. Also, emission intensity increased by 7% per kg of DMI (23.5 ± 0.43 g CH_4) and by 9% per unit of gross energy intake (GEI; 7.0 ± 0.14% CH_4) with grass maturity, implying an increased loss of dietary energy with progressing grass maturity. Rate of N fertilisation had no effect on CH_4 emission per units of DMI, GEI and FPCM. These results suggest that despite a lower absolute daily CH_4 production with a higher N fertilisation rate, CH_4 emission intensity remains unchanged. A significant reduction of CH_4 emission intensity can be achieved by feeding dairy cows silage of grass harvested at an earlier stage of maturity.

Key words: nitrogen fertilisation; grass maturity; ryegrass; methane; dairy cow

INTRODUCTION

Methane (CH_4) production by ruminant livestock has received considerable attention in recent years due to its contribution to greenhouse gas emission (IPCC, 2008). Moreover, enteric CH_4 formed from the fermentation of feed in the ruminant gastrointestinal tract implies a loss of digested energy (Johnson and Johnson, 1995). It is known that rumen hydrogen balance and CH_4 production is affected by diet degradability and diet chemical composition (for a recent review see Hristov et al., 2013). Grass silage is commonly fed in intensive dairy production and, hence, contributes to a main part of the CH_4 emitted. However, quantitative in vivo information on the effect of a range of grass silage qualities on CH_4 emissions is scarce.

Hammond et al. (2009) showed that variation in the chemical composition of ryegrass explained only a minor part of the in vivo variation in CH_4 production by dairy cows, and variation in digestibility particularly needs to be taken into account. Actual data on CH_4 formation per unit of digestible feed intake for grass silage-fed dairy cows are limited. A recent study with lactating cows indicated a substantial reduction (−15%) in CH_4 yield per unit of digestible organic matter intake (DOMI) with early-cut compared with late-cut (cut 3 weeks later) ryegrass-clover silage (Brask et al., 2013). Similar findings were reported for beef and dairy cattle fed various grass hay qualities (Boadi and Wittenberg, 2002).

Yet, a comprehensive in vivo study on the combined effect of N fertilisation rate of grassland and the maturity of the grass harvested for silage making on CH_4 emission by dairy cows fed grass silage is lacking. The objective of this study was thus to evaluate the effects of N fertilisation rate and maturity of grass harvested for silage making on CH_4 emission, feed intake, milk production, diet digestibility and energy balance of lactating cows. Our hypotheses were that an increasing N fertilisation rate increases the N content and digestibility of grass and that a decreasing grass maturity decreases fibre content and increases digestibility of grass, and CH_4 emission intensity is thus reduced.

MATERIALS AND METHODS

Experimental setup and dietary treatments

The experiment was conducted over six weeks from January to March 2013 at the animal research facilities Carus of Wageningen University, Wageningen, The Netherlands, and was approved by the Institutional Animal Care and Use Committee of Wageningen University. In

a completely randomised block design with six dietary treatments, 54 lactating Holstein-Friesian dairy cows (12 primiparous and 42 multiparous) were used.

Treatments consisted of grass swards fertilised at a low rate (LF; 65kg of N/ha) and a high rate (HF; 150 kg of N/ha), and harvested at three stages of grass maturity: early (EM; after 28 days of regrowth at a targeted yield of 2000 kg of DM/ha), mid (MM; after 41 days of regrowth) and late maturity (LM; after 62 days of regrowth) (Table 1). Cows were assigned to 9 blocks (6 cows per block) based on parity, lactation stage (168 ± 11 days in milk; mean ± S.E.), expected milk yield, and whether cows were rumen cannulated or not. Twelve cows previously fitted with a permanent rumen cannula (10 cm i.d., Type 1C; Bar Diamond Inc., Parma, ID, USA) were used for rumen fluid collection. Within blocks, cows were randomly assigned to one of six dietary treatments.

Grassland management

The grass originated from a mixed sward on sandy soil established in 2010 and composed of diploid perennial ryegrass (*Lolium perenne*) cultivars of intermediate- and late-heading type (36% each) and timothy (*Phleum pratense*; 28%) (BG Superplus, Barenbrug, Oosterhout, The Netherlands). Grass swards were grown on three fields and treatments were randomly distributed across fields. Grass swards received an initial 80 kg of N/ha from cattle slurry, followed by either 45 or 115 kg of N/ha (LF and HF, respectively) from calcium ammonium nitrate (Rijnvallei, Wageningen, The Netherlands). The first cut did not result in satisfactory differences in N content of grass among treatments nor sufficient DM yields because of unfavourable weather conditions. Therefore, all fields were cut on 22 May 2012 and the fertilisation regimen for the regrowth swards was increased to 65 and 150 kg of N/ha (LF and HF, respectively) from calcium ammonium nitrate, which was applied a week after harvest. Grass was harvested either on 19 June 2012 (EM), 2 July 2012 (MM) or 23 July 2012 (LM) at approximately the same time (ca. 1500 h), wilted on field for approximately 24 h and ensiled in bales (ca. 500 kg) using 12 layers of stretch plastic without addition of inoculants. Average daily temperature was 16.3°C (SD 2.9°C) and precipitation totalled 191 mm during the entire regrowth period from May to July 2012.

Feeding and housing

Cows were fed a diet consisting of 80% grass silage and 20% compound feed on DM basis (Table 1) in two equal daily portions at 0600 and 1600 h. The compound feed was provided separately as a meal to ensure its complete intake. The external marker chromium

Table 1. Chemical composition of grass silages harvested with a low and high nitrogen fertilisation (F) rate (65 kg/ha, LF; and 150 kg/ha, HF; respectively) at an early (EM; 28 days of regrowth), mid (MM; 41 days of regrowth) and late (LM; 62 days of regrowth) stage of grass maturity at cutting, and of compound feed.

	Grass silage						
	LF			HF			Cpd
Item	EM	MM	LM	EM	MM	LM	feed[1]
DM yield (kg/ha)	2,023	3,214	3,535	2,055	3,609	5,793	—
Chemical characteristics[2]							
DM (g/kg of product)	436	654	762	430	575	540	937
Organic matter	903	924	934	895	902	914	907
CP	149	106	78	197	173	120	469
Crude fat	33	27	22	35	33	25	33
Starch[3]	—	—	—	—	—	—	158
Sugars	98	190	179	54	79	69	106
NDF	476	501	561	459	507	603	93
ADF	282	288	315	280	298	353	44
ADL	20	24	26	21	22	32	3
GE (kJ/g of DM)	19.2	18.7	18.4	19.3	19.1	18.9	17.8
DVE[4]	56	64	51	65	71	45	242
OEB[4]	40	−24	−44	93	31	7	192
Silage characteristics[2,4]							
pH	5.1	5.5	5.9	4.9	5.6	5.3	—[5]
Ammonia-N (% of total N)	10.5	4.0	3.8	11.8	5.5	9.5	—
Acetic acid	14.3	11.5	21.3	23.8	10.5	19.3	—
Lactic acid	33.0	27.8	18.5	43.3	18.8	32.3	—
Nitrate	1.7	0.4	0.5	6.2	4.3	1.5	—

[1]Compound feed. Ingredients (per kg of DM): soybean meal (412 g), rumen-protected soybean meal (234 g; Rumi-S, Schouten Products, Rotterdam, The Netherlands), maize (218 g), beet molasses (60 g), urea (31 g), limestone (23 g), calcined magnesia (8.4 g), calcium biphosphate (7.3 g), rumen-inert palm fat (3.9 g; Hidropalm, Norel, Madrid, Spain), chromium oxide (3.3 g).

[2]Values in g/kg of DM, unless specified otherwise.

[3]Starch was not determined in the grass silage.

[4]DVE = Intestinal digestible protein; OEB = Rumen-degraded protein balance. Determined by NIRS for grass silages (BLGG AgroXpertus, Wageningen, The Netherlands); determined from Dutch feed table values for compound feed.

[5]Not relevant.

oxide (2,276 mg of chromium/kg of DM) was added to the compound feed (Research Diet Services, Wijk bij Duurstede, The Netherlands) for estimation of apparent total-tract feed digestibility. The grass silage was prepared twice weekly using a self-propelled mixer wagon (Strautmann Verti-Mix 500, Bad Laer, Germany) equipped with a cutter loader system and an electronic weighing scale and stored at 6°C. Water was offered ad libitum during the entire experiment.

Cows were individually housed in tie-stalls for 12 days to adapt to the corresponding experimental diet. Cows were fed ad libitum for the first 8 days. From day 9 onwards and before cows were moved to the climate respiration chambers (CRC), feed intake was restricted per block to 95% based on the ad libitum dry matter intake (DMI) of cows with the smallest DMI within a block. Feed restrictions were based on intake records from days 3 through 8 as described by Van Zijderveld et al. (2011) but at all times a minimum DMI of at least 80% of the ad libitum intake of cows with the greatest DMI within block were ensured. Because each block consisted of six cows but the four CRC units offered space for testing a maximum of four cows simultaneously, feed restriction was based on the smallest DMI of four cows per block.

On day 13 (1500 h), cows were moved individually to one of four identical CRC located in close vicinity of the tie-stall barn. Each block of six cows was completed within two consecutive periods; cows and treatments were randomly distributed to the CRC and periods. A detailed description of the CRC design and gas measurements was reported by Van Gastelen et al. (2015). Briefly, in each CRC (volume 35 m^3) relative humidity was maintained at 70% and temperature at 16°C. The ventilation rate in each compartment was 42 m^3/h. Inlet and exhaust air of each compartment was sampled at 10-min intervals. Gas concentrations and ventilation rates were corrected for pressure, temperature and humidity to arrive at standard temperature pressure dew point volumes of inlet and exhaust air. Cows were exposed to 16 h of light per day. Cows stayed in the CRC until day 17 (0900 h). Methane measurements were based on data recorded from day 14 (0800 h) through day 17 (0800 h); energy balance measurements were based on total manure collection from day 13 (1500 h) through day 17 (0900 h).

Sample collection and measurements

On day 10 and 11, rumen fluid samples (~350 ml) were collected in duplicates and proportionally from a cranial, middle and caudal direction from 12 rumen-cannulated cows, pertaining to blocks 1 and 9, at times t = 0, 1, 2, 3, 4, 6, 8 and 10 h after morning feeding

using a perforated plastic tube (2.5 cm i.d.). Rumen pH was measured immediately using a mobile electronic pH meter (pH electrode HI1230, Hanna Instruments, IJsselstein, The Netherlands). Subsamples of 50 ml were immediately stored at −20°C pending ammonia-N analysis, and subsamples of 750 µl were acidified with an equal volume of ortho-phosphoric acid and stored at −20°C pending volatile fatty acid (VFA) analysis.

Milk from cows in the CRC was collected twice daily at 0600 and 1600 h. A milk sample (10 ml) of each milking was collected in a tube containing 5 µl NaN_3 for preservation for analyses of milk fat, protein and lactose. Milk composition reported was corrected for differences in milk yield between individual milking events. Additional milk samples (5 g/kg of milk) were taken at each milking, pooled per cow and stored −20°C for milk energy analyses. For energy balance determination, faeces and urine from the CRC were quantitatively collected as a mixture, homogenised and subsamples of ~500 ml from the mixture were stored at −20°C. For estimation of apparent total-tract feed digestibility, grab samples of faeces were collected twice daily during milking times at 0600 and 1600 h, pooled per cow and stored at −20°C. Feed residues were collected each morning, pooled per cow and stored at −20°C pending analyses. Samples were taken from feed portions offered to cows in the CRC and pooled per cow. Rumen degradation characteristics of the grass silages used in the present study were determined in a separate experiment using in situ nylon bag incubations, and results were reported by Heeren et al. (2014).

Chemical analysis

Samples of feed, feed residues, manure and faecal grab samples collected in the CRC from day 13 through 17 were oven-dried at 60°C, ground to pass a 1-mm sieve (Peppink 100 AN, Peppink, Olst, The Netherlands), and analysed by wet chemistry according to the methods described in detail by Abrahamse et al. (2008). Gross energy (GE) was determined by bomb calorimetry after ISO 9831 (ISO, 1998). Grass silage and compound feed samples were analysed for DM, ash, N, crude fat, starch (compound feed only), sugars, NDF, ADF, ADL, and GE. Feed residues were analysed for DM and ash. Faecal grab samples and manure samples were analysed for DM, ash, crude fat, NDF and GE. Chromium was determined in compound feed and faecal grab samples by using an atomic absorption spectrophotometer (AA240FS, Varian, Palo Alto, CA, USA) after oxidation with wet destruction as described in detail by Pellikaan et al. (2013).

The concentration of individual VFA was determined using gas chromatography (GC type Fisons HRGC MEGA2, Milan, Italy) as described by Warner et al. (2013a), the

nonglucogenic-to-glucogenic VFA ratio (NGR; i.e. [acetate + 2 × butyrate + 2 × isobutyrate + valerate + isovalerate]/[propionate + valerate + isovalerate]) was calculated after Abrahamse et al. (2008). And that of ammonia-N using the indophenol reaction as described by Searle (1984). Milk composition was analysed by mid-infrared spectroscopy at VVB Milk Control Station (Nunspeet, The Netherlands) using the standard procedure ISO 9622 (ISO, 1999), and GE content in milk was analysed as described above.

Statistical analysis

Data on feed intake, milk production and composition were pooled per cow. Data were subjected to ANOVA in a randomised block design with a 2 × 3 factorial arrangement of treatments (2 N fertilisation rates × 3 grass maturity stages) by mixed model procedures of SAS (version 9.3, SAS Institute, Cary, NC, USA). Treatments and their interactions were considered fixed effects and blocks were considered random effects. Each block of six cows, each was completed within two consecutive periods because only four cows could be contemporaneously housed in the CRC units. Per block, cows and treatments were randomly assigned to one of two consecutive periods. Therefore, period effect was confounded with block effect. Covariance parameters were estimated using the REML method with variance component structure as the covariance structure, and denominator degrees of freedom were estimated using the Kenward-Roger approximation. Data from two cows (treatments HF–EM and HF–LM) were removed from statistical analyses on measurements taken in the CRC due to outlying values (considered here as more than 5 × S.E.) observed for DMI and milk production. Pairwise comparisons of means were tested with the Tukey-Kramer method. Rumen fluid measurements from two sampling days were pooled and data were subjected to repeated measures ANOVA to take repeated samples over time within the same cow into account. Because of unequal sampling time intervals, spatial power variance components were used as the covariance structure to account for within-cow variation.

All results are reported as least square means with significance of effects declared at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$.

RESULTS

Feed intake and milk yield

Compound feed was fed before the grass silage was offered and was generally completely consumed. Hence, differences in intake levels of DM, nutrients and the compound feed proportion presented in Table 2 were caused by differences in the intake of the respective

Table 2. Feed intake of dairy cows fed rations containing grass silage with a low and high nitrogen fertilisation (F) rate (65 kg/ha; LF and 150 kg/ha, HF; respectively) and harvested at an early (EM; 28 days of regrowth), mid (MM; 41 days of regrowth) and late (LM; 62 days of regrowth) stage of grass maturity (M) at cutting[1].

	LF			HF				*P*-value		
Item	EM	MM	LM	EM	MM	LM	S.E.	F	M	F × M
Intake (kg/d)										
Dry matter	15.8	14.9	14.9^{X}	16.0^{a}	14.5^{b}	$13.3^{c,Y}$	0.56	0.009	≤0.001	0.004
Organic matter	14.2	13.8	13.8^{X}	14.4^{a}	13.1^{b}	$12.2^{b,Y}$	0.52	≤0.001	≤0.001	0.002
CP	$3.3^{a,X}$	$2.7^{b,X}$	$2.3^{c,X}$	$3.9^{a,Y}$	$3.4^{b,Y}$	$2.7^{c,Y}$	0.11	≤0.001	≤0.001	0.004
DOM	11.5	10.9	10.2^{X}	11.6^{a}	10.5^{a}	$8.8^{b,Y}$	0.42	0.004	≤0.001	0.009
DNDF	4.9	4.8	4.6	5.0	4.9	4.3	0.24	0.701	0.015	0.413
Compound feed[2] (%)	19.3	20.4	20.6^{X}	19.0^{a}	21.1^{a}	$23.1^{b,Y}$	0.46	0.005	≤0.001	0.002

DOM = digestible organic matter; DNDF = digestible NDF; S.E. = standard error of the means.

[1]For a significant treatment interaction (F × M) values within a row with different lower-case superscript letters differ significantly at $P \leq 0.05$ within F, and values within a row with different upper-case superscript letters differ significantly at $P \leq 0.05$ within M.

[2]Proportion of compound feed in the ration (rest is grass silage).

grass silages. Occasional feed residues pertained to grass silage and were largest for cows receiving the HF–LM grass silage. Hence, these cows consumed a somewhat larger proportion of compound feed. Although DMI was restricted, DMI decreased by 4% with N fertilisation ($P = 0.009$) and by 11% with grass maturity ($P \leq 0.001$). Fertilisation effects were particularly observed for LM cuts, and maturity effects were particularly observed for HF grass (significant treatment interaction; $P = 0.004$). A similar pattern was observed for organic matter intake (OMI), digestible OMI, and a pattern in opposite direction for the compound feed proportion in the diet. Intake of DNDF decreased with grass maturity ($P = 0.015$) but was not affected by N fertilisation rate.

Yields of milk ($P \leq 0.001$), FPCM ($P \leq 0.001$), milk fat ($P \leq 0.001$) and milk protein ($P \leq 0.001$) decreased with grass maturity but no N fertilisation effects occurred (Table 3). Yield of FPCM and yield of fat decreased with grass maturity on average by 26% and 28%, respectively. For LF grass, maturity effects were observed between EM and MM cuts, whereas for HF grass, maturity effects were observed only between MM and LM cuts. This resulted in a significant treatment interaction for FPCM ($P = 0.040$) and milk fat ($P = 0.010$) yields. Milk composition was not influenced by dietary treatments.

Feed digestibility and rumen fermentation

Apparent total-tract digestibility of all chemical fractions as well as GE decreased with grass maturity ($P \leq 0.001$), and N ($P \leq 0.001$) and crude fat ($P = 0.032$) digestibility was higher with HF grass (Table 4). Rumen ammonia-N concentrations were generally greater with HF compared with LF grass silage ($P \leq 0.001$), and were greater with reduced grass maturity ($P = 0.006$) (Table 5). Propionate molar proportion increased with maturity ($P \leq 0.001$) but only when feeding LF grass silage ($P = 0.025$). Valerate ($P \leq 0.001$) and branched-chain VFA ($P = 0.031$) molar proportions decreased with grass maturity in line with a decreasing NGR with N fertilisation ($P = 0.029$). Acetate and butyrate molar proportions were not affected by dietary treatments. Sampling time had a significant effect on rumen fluid characteristics. Molar proportions of acetate decreased within the first 2 h after morning feeding, whereas those of propionate, butyrate, valerate and branched-chain VFA initially increased to approach baseline values again at 10 h after morning feeding (data not shown); ammonia-N concentrations were greatest at the first sampling (1 h) after morning feeding and decreased continuously thereafter (data not shown).

Table 3. Milk yield and composition of dairy cows fed rations containing grass silage with a low and high nitrogen fertilisation (F) rate (65 kg/ha, LF; and 150 kg/ha, HF; respectively) and harvested at an early (EM; 28 days of regrowth), mid (MM; 41 days of regrowth) and late (LM; 62 days of regrowth) stage of grass maturity (M) at cutting[1].

	LF			HF				*P*-value		
Milk	EM	MM	LM	EM	MM	LM	S.E.	F	M	F × M
Yield (kg/d)										
Milk	26.0	21.5	20.6	25.4	23.6	18.7	1.87	0.893	≤0.001	0.228
FPCM	28.8[a]	22.4[b]	21.4[b]	26.6[a]	25.3[a]	19.7[b]	1.57	0.713	≤0.001	0.040
Fat	1.29[a]	0.93[b]	0.90[b]	1.14[a]	1.10[a]	0.84[b]	0.069	0.700	≤0.001	0.010
Protein	0.84	0.73	0.67	0.81	0.77	0.61	0.040	0.488	≤0.001	0.211
Composition (g/kg)										
Fat	50.0	44.3	45.2	45.0	48.0	45.7	2.42	0.870	0.583	0.092
Protein	32.7	34.5	34.4	32.6	33.4	33.5	1.74	0.537	0.525	0.934
Lactose	46.1	44.0	45.3	45.2	45.2	44.7	0.76	0.873	0.250	0.202

FPCM = fat- and protein-corrected milk; S.E. = standard error of the means.

[1]For a significant treatment interaction (F × M) values within a row with different lower-case superscript letters differ significantly at $P \leq 0.05$ within F.

Table 4. Apparent total-tract digestibility in dairy cows fed rations containing grass silage with a low and high nitrogen fertilisation (F) rate (65 kg/ha, LF; and 150 kg/ha, HF; respectively) and harvested at an early (EM; 28 days of regrowth), mid (MM; 41 days of regrowth) and late (LM; 62 days of regrowth) stage of grass maturity (M) at cutting[1].

	LF			HF				*P*-value		
Digestibility (%)	EM	MM	LM	EM	MM	LM	S.E.	F	M	F × M
Organic matter	80.6	79.5	73.9	80.3	80.0	72.1	0.73	0.318	≤0.001	0.234
Nitrogen	75.5^{a}	70.7^{b,X}	69.9^{b,X}	77.6	77.0^{Y}	75.3^{Y}	0.78	≤0.001	≤0.001	0.015
Crude fat	71.3	58.2	54.9	69.8	65.0	61.3	2.36	0.032	≤0.001	0.113
NDF	77.9	76.0	66.4	79.1	79.7	65.6	1.32	0.179	≤0.001	0.202
Gross energy	78.5	76.3	70.8	78.0	77.2	69.6	0.81	0.687	≤0.001	0.333

S.E. = standard error of the means.

[1]For a significant treatment interaction (F × M) values within a row with different lower-case superscript letters differ significantly at $P \leq 0.05$ within F, and values within a row with different upper-case superscript letters differ significantly at $P \leq 0.05$ within M.

Table 5. Rumen fermentation characteristics of dairy cows fed rations containing grass silage with a low and high nitrogen fertilisation (F) rate (65 kg/ha, LF; and 150 kg/ha, HF; respectively) and harvested at an early (EM; 28 days of regrowth), mid (MM; 41 days of regrowth) and late (LM; 62 days of regrowth) stage of grass maturity (M) at cutting[1].

	LF			HF				*P*-value[2]		
Item	EM	MM	LM	EM	MM	LM	S.E.	F	M	F × M
pH	6.6	6.3	6.4	6.6	6.5	6.5	0.09	0.124	0.072	0.378
Ammonia-N (mmol/l)	16.4	12.1	10.0	22.8	19.0	16.3	1.58	≤0.001	0.006	0.973
Total VFA (mmol/l)	101	109	109	104	112	113	3.5	0.282	0.069	0.973
VFA (mol/100 mol)										
Acetate	68.5	68.0	67.8	66.4	66.6	68.2	0.83	0.151	0.688	0.343
Propionate	15.4[a]	17.6[b]	18.0[b]	16.8[a]	17.7[a]	17.1[a]	0.38	0.577	≤0.001	0.025
Butyrate	11.1	11.6	11.4	11.7	10.9	10.8	0.54	0.559	0.850	0.448
Valerate	1.92	1.17	1.13	1.87	1.55	1.40	0.081	0.014	≤0.001	0.055
Branched-chain VFA	3.03	1.57	1.64	3.28	3.31	2.54	0.318	0.007	0.031	0.133
NGR	5.1	4.9	4.7	4.7	4.5	4.7	0.13	0.029	0.212	0.200

VFA = volatile fatty acids; NGR = nonglucogenic-to-glucogenic VFA ratio; S.E. = standard error of the means.

[1]For a significant treatment interaction (F × M) values within a row with different lower-case superscript letters differ significantly at $P \leq 0.05$ within F.

[2]Effect of sampling time (0, 1, 2, 3, 4, 6, 8 and 10 h after morning feeding) was significant ($P \leq 0.001$) for all rumen fermentation parameters, interaction (sampling time × F × M) was not significant ($P > 0.05$).

Methane production and energy balance

Daily CH_4 production decreased by 4% with N fertilisation ($P = 0.035$) and by 6% with grass maturity ($P = 0.024$) (Table 6). Enteric CH_4 emission intensity was not affected by N fertilisation but generally increased with grass maturity ($P \leq 0.001$). Methane emission expressed per unit of DMI increased by 7% with grass maturity ($P \leq 0.001$) but a significant increase occurred only for HF grass silage as indicated by a significant interaction between N fertilisation and grass maturity ($P = 0.018$). Methane emission per unit of FPCM increased by 31%, per DOMI by 15% ($P \leq 0.001$) and per GEI by 9% ($P \leq 0.001$) with grass maturity. No treatment effects were observed for CH_4 emission per unit of digestible NDF intake (DNDFI).

Levels of GEI ($P \leq 0.001$), digestible energy intake (DEI; $P \leq 0.001$) and metabolizable energy intake (MEI; $P \leq 0.001$) decreased with grass maturity but were comparable between N fertilisation rates (Table 7). A smaller amount of energy was lost to CH_4 ($P = 0.017$), heat and milk ($P \leq 0.001$), and diet metabolizability ($P \leq 0.001$) declined with progressing grass maturity. Energy lost to CH_4 tended ($P = 0.090$) to be lower for HF grass silage.

DISCUSSION

The objective of this study was to assess CH_4 emission by lactating dairy cows fed diets containing a large proportion of grass silage harvested under varying management conditions. Yet, in vivo data on combined effects of the rate of N fertilisation of grassland and the maturity of grass at harvest on enteric CH_4 production of grass silage-fed dairy cows have not been reported in literature.

In line with expectations from a previous grass silage trial (Warner et al., 2013b), the six grass silages tested varied in nutrient composition and digestibility, with HF grass silage containing more CP and less sugar than LF grass silage, and with progressing grass maturity resulting in decreased CP and increased sugar and fibre contents. Sugar content was greater for MM, probably related to weather conditions at harvest and wilting. Silage characteristics were not always in line with expectations because of difficult harvesting and wilting conditions as shown by somewhat high silage pH values and low concentrations of lactic acid compared with those of acetic acid. In addition, LM-LF grass silage had a particular high DM content.

Grass silage quality influenced enteric CH_4 emission depending on the unit in which CH_4 emission is expressed. Daily CH_4 production decreased with a higher N fertilisation rate and grass maturity by 4 and 6%, respectively. Although the amount of daily CH_4 produced per

Table 6. Methane production from dairy cows fed rations containing grass silage with a low and high nitrogen fertilisation (F) rate (65 kg/ha, LF; and 150 kg/ha, HF; respectively) and harvested at an early (EM; 28 days of regrowth), mid (MM; 41 days of regrowth) and late (LM; 62 days of regrowth) stage of grass maturity (M) at cutting[1].

	LF			HF				*P*-value		
Methane production	EM	MM	LM	EM	MM	LM	S.E.	F	M	F × M
CH_4 (g/d)	361	356	347	347	352	322	13.6	0.035	0.024	0.430
CH_4 (g/kg of DMI)	23.0	24.0	23.4	21.7^{a}	24.4^{b}	24.6^{b}	0.43	0.848	≤0.001	0.018
CH_4 (g/kg of DOMI)	31.6	32.5	33.9^{X}	30.1^{a}	33.6^{b}	36.9^{c,Y}	0.78	0.165	≤0.001	0.018
CH_4 (g/kg of DNDFI)	73.6	75.0	75.8	70.5	72.5	77.5	2.74	0.548	0.230	0.623
CH_4 (% of GEI)	6.8	7.2	7.1	6.4	7.2	7.3	0.14	0.493	≤0.001	0.078
CH_4 (g/kg of FPCM)	12.8	16.0	16.8	13.2	14.0	17.4	1.00	0.611	≤0.001	0.260

DMI = dry matter intake; DOMI = digestible organic matter intake; DNDFI = digestible NDF intake; GEI = gross energy intake; FPCM = fat- and protein-corrected milk; S.E. = standard error of the means.

[1]For a significant treatment interaction (F × M) values within a row with different lower-case superscript letters differ significantly at $P \leq 0.05$ within F, and values within a row with different upper-case superscript letters differ significantly at $P \leq 0.05$ within M.

Table 7. Energy balance in dairy cows fed rations containing grass silage with a low and high nitrogen fertilisation (F) rate (65 kg/ha, LF; and 150 kg/ha, HF; respectively) and harvested at an early (EM; 28 days of regrowth), mid (MM; 41 days of regrowth) and late (LM; 62 days of regrowth) stage of grass maturity (M) at cutting.

Energy balance (kJ/kg of $BW^{0.75}$ per day)	LF			HF			S.E.	*P*-value		
	EM	MM	LM	EM	MM	LM		F	M	F × M
BW ($kg^{0.75}$)	124	124	126	123	125	124	3.0	0.633	0.650	0.670
GEI	2,416	2,229	2,168	2,484	2,186	2,000	68	0.243	≤0.001	0.071
DEI[1]	1,898	1,702	1,536	1,939	1,688	1,395	59	0.333	≤0.001	0.163
MEI[2]	1,654	1,423	1,319	1,714	1,424	1,235	61	0.876	≤0.001	0.428
MEI:GEI ratio (%)	68.5	64.1	61.0	69.1	65.2	61.8	1.68	0.529	≤0.001	0.990
MEI:DEI ratio (%)	87.2	82.3	86.1	88.6	84.3	88.7	2.11	0.215	0.050	0.954
Energy in methane	163	160	154	158	157	144	4.9	0.090	0.017	0.706
Energy in milk	719	556	521	672	625	495	36.6	0.958	≤0.001	0.101
Heat production	899	847	784	908	854	792	19.9	0.539	≤0.001	0.999
Energy retention[3]	36	25	14	135	−58	−47	63.2	0.757	0.161	0.266

GEI = gross energy intake; DEI = digestible energy intake; MEI = metabolizable energy intake; S.E. = standard error of the means.

[1]Calculated as GEI × GE digestibility.

[2]Calculated as GEI − energy in manure − energy in methane.

[3]Calculated as MEI – heat production – energy in milk.

cow with LM was smaller than with EM grass silage, CH_4 emission per unit of FPCM and DOMI was larger because of lower feed digestibility and FPCM production with LM grass silage. Such an increase in CH_4 emission intensity is in line with results of Brask et al. (2013) obtained with early first cut versus late first cut ryegrass-clover silage.

A part of the N fertilisation effect on CH_4 production was likely due to the relatively high nitrate content of the grass silages tested in the present study. The nitrate supply was particularly large for EM and MM of HF grass silages (80 and 49 g of nitrate/day, respectively). This nitrate has likely served as a hydrogen sink in the rumen and resulted in a reduced CH_4 production (Navarro-Villa et al., 2011). Assuming a stoichiometric CH_4 reduction of 0.258 g with every gram nitrate in the diet (Van Zijderveld et al., 2010), CH_4 production per cow was likely reduced by 21 g/day (6%) and 13 g/day (4%), respectively, for the treatments high in nitrate (HF–EM and HF–MM), whereas only a minor CH_4 reduction of less than 6 g/day occurred for the LF grass silages low in nitrate. We can thus speculate that differences in CH_4 production between HF and LF grass are negligible in a situation with low-nitrate grass silages.

Methane production in relation to milk output

Enteric CH_4 emission per unit of FPCM increased considerably with grass maturity (+31%), in line with a reduced FPCM production (−26%). A clear increase in CH_4 emission intensity may be also calculated for late-cut relative to early-cut ryegrass-clover silage (+15%; Brask et al., 2013). Our findings are consistent with a model-simulation study by Bannink et al. (2010) in which CH_4 per unit of FPCM was predicted to be 10% higher for late-cut (4500 kg of DM/ha) relative to early-cut (3000 kg of DM/ha) ryegrass silage. The model-simulation study further predicted an effect of N fertilisation rate with a 13% reduction for a high (350 kg of N/ha per year) relative to a low (150 kg of N/ha per year) fertilisation regimen. The present in vivo study did not confirm this large fertilisation effect on CH_4 emission intensity. However, it should be noted that the grass silages tested in the present study were harvested at a slightly greater DM yield, had a higher sugar content, a lower CP content and a lower rumen degradability (Heeren et al., 2014) than assumed by Bannink et al. (2010). This may explain the relatively small amount of CH_4 per unit of FPCM with LF grass silage and, thus, explain the similar CH_4 emission levels between N fertilisation rates in the present study in contrast to the differences simulated by Bannink et al. (2010).

Grass silage quality effects described in the present study were generally in line with those observed for dairy cows offered grass herbage under zero-grazing conditions (Warner et

al., 2015). Increasing grass maturity from 3 to 5 weeks of regrowth increased CH_4 per unit of FPCM by 14%, whereas increasing the N fertilisation rate from 20 to 90 kg of N/ha did not affect CH_4 emission. The grass silage tested in the present study generated 8% more daily CH_4 per cow than the grass herbage tested by Warner et al. (2015). Nonetheless, emitted CH_4 per unit of FPCM was slightly lower (−4%) in the present study due to a considerably larger milk production (+16%) realised with grass silage at comparable DMI levels.

Methane production in relation to feed intake and digestibility

In line with a reduction in DMI, enteric CH_4 production per unit of DMI increased by 7% with grass maturity. Largely this was caused by a relatively small DMI with HF–LM grass silage because an increased feed intake level generally coincides with shorter retention times in the rumen and lesser amounts of CH_4 per unit feed (Hristov et al., 2013). It should be however noted that the proportion of compound feed in the total diet was larger with HF–LM grass silage and this greater compound feed uptake may reduce CH_4 emission in high-producing dairy cows through an increase in propionate (Boadi et al., 2004). However, for this treatment, we observed no particular change in propionate molar proportions or in starch intake (data not shown) which may induce a shift in rumen VFA towards more propionate. Thus, the low daily CH_4 production for low-digestible HF–LM grass silage was most likely related to a particular small DMI.

The increasing propionate molar proportions with advancing silage maturity for LF grass was not expected but may be explained by the relatively high sugar content. In contrast, no significant increase in propionate molar proportions with silage maturity occurred with HF grass. Likely, the unexpected higher acetate:propionate ratio with EM–HF and MM–HF grass silages high in nitrate was induced by nitrate reduction, which decreased the amount of hydrogen in the rumen otherwise used for propionogenesis (Farra and Satter, 1971) and methanogenesis (previously discussed).

Apparent total-tract digestibility decreased as expected with progressing grass maturity for all nutrients and for GE, in line with Brask et al. (2013), except for crude fat digestibility, which increased with maturity in the latter study. A lower crude fat digestibility with advancing maturity may be explained by lower crude fat content of late-cut grass, as endogenous fat secretions have a relatively larger impact on apparent digestibility at low fat content. Total-tract digestibility was unaffected by N fertilisation, except for a higher N and crude fat digestibility with HF compared with LF grass silage. In line with observed in vivo digestibility, in situ rumen degradability of the same grass silage (Heeren et al., 2014) was

reduced with progressing grass maturity or reduced N fertilisation rate (except for OM in agreement with the absence of an effect of N fertilisation rate on in vivo OM digestibility). Whereas effects of N fertilisation on in situ rumen degradability generally depended on the grass maturity stage (Heeren et al., 2014), such a treatment interaction effect did not occur for in vivo digestibility in the present study except for N digestibility.

When expressed per unit of digestible nutrient intake, CH_4 production increased by 15% per unit of DOMI with progressing grass maturity but did not change per unit of DNDFI. A similar large increase of 18% was reported for a late-cut compared with early-cut (cut 3 weeks earlier) ryegrass-clover silage (Brask et al., 2013). No further in vivo data are available to support our findings on grass silage-fed dairy cows. However, the present results are in line with a study of Boadi and Wittenberg (2002) in which medium-quality grass hay (50.7% in vitro OM digestibility) generated 10% more CH_4 per unit of DOMI than high-quality grass hay (61.5% in vitro OM digestibility) offered restrictedly to dairy and beef heifers, and in line with a study of Pinares-Patino et al. (2003) in which Charolais cows grazing timothy herbage at heading stage (74.8% OM digestibility based on faecal N index method) produced 12% more CH_4 per unit of DOMI than when grazing early vegetative stage timothy herbage (77.6% OM digestibility). In contrast, when dairy cows were offered grass herbage under zero-grazing conditions (Warner et al., 2015), CH_4 per unit of DOMI was not affected by either grass maturity or N fertilisation rate, whereas CH_4 declined by 9% per unit of DNDFI with high- compared with low-fertilised grass (90 vs. 20 kg of N/ha, respectively). Despite a clear reduction in NDF digestibility by 12%-units with maturity in the present study, grass silage quality did not affect DNDFI levels and CH_4 per unit of DNDFI. Brask et al. (2013) reported similar findings on CH4 per unit DNDFI for ryegrass-clover silage.

Total CH_4 production (g/d) was well related to feed intake despite a restricted feeding regimen imposed on the experimental animals. In contrast, only a minor part of the variation in CH_4 production could be explained by feed OM digestibility (Figure 1) despite the range in grass digestibility over three maturity stages. This was also confirmed by the fact that DOMI did not improve the relationship between CH_4 production and OMI. These findings are in line with a study on timothy offered to Charolais cows at various stages of grass maturity (Pinares-Patino et al., 2003) and agree with the concept that intake is the main explanatory variable of CH_4 production (Ellis et al., 2007). Digestibility and (digestible) intake of OM was negatively related to CH_4 energy losses as % of GEI (Figure 1) or CH_4 emission per unit of

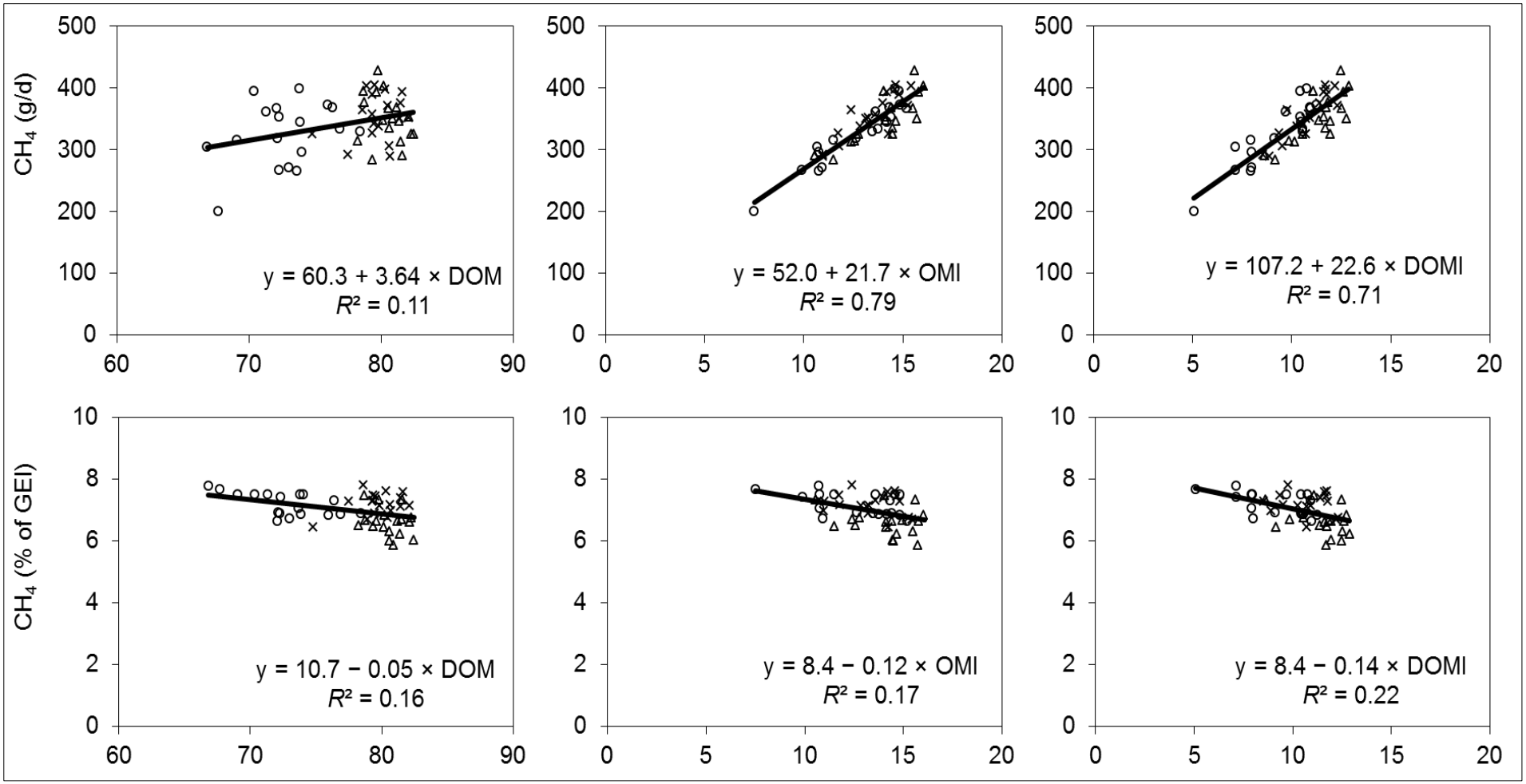

Figure 1. Linear relationships ($P \leq 0.05$) between enteric methane (CH_4) emission (g/d and % of gross energy intake (GEI)) and apparent total-tract organic matter digestibility (DOM; %), organic matter intake (OMI; kg/d) or digestible organic matter intake (DOMI; kg/d) from lactating dairy cows fed rations containing grass silages fertilised at 65 and 150 kg of N/ha, and harvested at an early (28 days of regrowth; triangle symbols △), mid (41 days of regrowth; cross symbols ✕) and late (62 days of regrowth; circle symbols ○) stage of grass maturity at cutting.

DMI (data not shown), but relationships were weak. Similar relationships were reported in other studies (Pinares-Patino et al., 2003; Hart et al., 2009).

IMPLICATIONS

The present study indicates that CH_4 emission intensity by lactating cows can be reduced by feeding less mature grass silage. Relationships between diet digestibility (mainly conditioned by grass maturity) and CH_4 emission were at the most moderate. However, effects were likely partly concealed by differences in feed intake and chemical composition, resulting from a different N fertilisation, stage of maturity at harvest, growing, and harvesting conditions. On a whole-farm scale, these CH_4 mitigating effects are most likely increased because OM fermentation and related CH_4 emission during manure storage will be smaller with higher digestible grass silage (Montes et al., 2013). Because an earlier grass harvest improves grass digestibility without the need of applying additional N fertilisers, potential sources of N_2O or CO_2 from application of artificial N fertilisers will not accrue and greenhouse gas emissions can be efficiently reduced with no additional N inputs. With an earlier grass harvest care should be taken that rumen fermentation is not impaired by deficient fibre intake, and that a larger N excretion into the environment through increased grass CP intake may potentially offset any benefits from reduced CH_4 emissions. The smaller herbage yields with an earlier grass harvest should be further taken into account. A holistic approach in form of a life-cycle assessment will provide a better assessment of the environmental impact and mitigation options for reducing greenhouse gas emissions from improved forage quality (Gerber et al., 2010). Van Middelaar et al. (2014) evaluated greenhouse gas emissions (emission of CO_2, N_2O and CH_4) from dairy farming at the chain level (i.e., from production of farm inputs to the farm gate) of several feeding strategies based on a life-cycle assessment. In this integrated approach, they calculated reduced GHG emissions per kg FPCM upon reducing the maturity stage of fresh and ensiled grass, and this strategy also was the most cost-effective.

Our results further suggest that increasing the N fertilisation rate in the present range of fertilisation rates and growing conditions of grass was ineffective in reducing enteric CH_4 emission from dairy cows fed this grass silage. Although it was not measured in this study, our results imply that reducing the amount of N fertiliser within the range tested for the purpose of reducing N excretion into the environment does not necessarily increase CH_4 emissions. Given the limited effect of N fertilisation on grass digestibility despite application of the relatively low and high N fertilisation rate used in the present study, further work is

needed to confirm the present results and to the effects of grass silage digestibility affected by variation in N fertilisation.

CONCLUSIONS

This study demonstrated that grass silage quality can affect CH_4 emission depending on the unit it is expressed. Daily CH_4 production per cow decreased with a higher N fertilisation rate and grass maturity. However, this drop in CH_4 production was offset by a lower FPCM production and total-tract digestibility, which resulted in a considerably greater CH_4 emission per unit of FPCM, DOMI and GEI with progressing grass maturity. Overall, these results suggest that a significant reduction of CH_4 emission intensity can be achieved by cutting grass at an earlier stage of maturity, whereas N fertilisation rate does not affect CH_4 emission intensity.

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CHAPTER 6

Diversity of condensed tannins structure in sainfoin (*Onobrychis viciifolia*) accessions affects in vitro rumen methane production

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ABSTRACT

Sainfoin is a non-bloating temperate forage legume with a moderate-to-high condensed tannin (CT) content. This study investigated whether the diversity of sainfoin accessions in terms of CT structures and contents could be related to rumen in vitro gas and methane (CH_4) production and fermentation characteristics. The aim was to identify promising accessions for future investigations. Accessions differed ($P < 0.001$) in terms of total gas and CH_4 productions. Fermentation kinetics (i.e., parameters describing the shape of the gas production curve and half-time gas production) for CH_4 production was influenced by accession ($P \leq 0.038$) but not by inclusion of polyethylene glycol (PEG), a tannin-binding agent. Accession, PEG and time ($P < 0.001$) affected CH_4 production, but accession and PEG interaction showed only a tendency ($P = 0.080$). Increase in CH_4 due to PEG addition was not related to CT content. Further analysis of the relationships among multiple traits (nutritional composition, CT structure and CH_4 production) using principal component analysis (PCA) based on optimally weighted variables revealed differences among accessions. The first two principal component axes, PC1 (57.6%) and PC2 (18.4%), explained 76.0% of the total variation among accessions. Loading of biplots derived from both PCAs made it possible to establish a relationship between the ratio of prodelphinidin:procyanidin (PD:PC) tannins and CH_4 production in some accessions. The PD:PC ratio seems to be an important source of variation that is negatively related to CH_4 production. These results suggested that sainfoin accessions collected from across the world exhibited substantial variation in terms of their effects on rumen in vitro CH_4 production revealing some promising accessions for future investigations.

Key words: sainfoin accession; methane; condensed tannins; prodelphinidin

INTRODUCTION

Sainfoin (*Onobrychis viciifolia*) is a perennial forage legume that is widely adapted to warm-temperate and dry land areas of Europe, Asia and the west of North America (Hayot Carbonero et al., 2011a). It can be grown as a pure pasture or mixed with companion grasses such as perennial ryegrass (*Lolium perenne*), meadow fescue (*Festuca pratensis*) and timothy (Phleum pratense) (Haering et al., 2008; Liu et al., 2010). It leads to higher intakes than lucerne when consumed by sheep (Khalilvandi-Behroozyar et al., 2010) and ruminants without causing bloat (Mueller-Harvey, 2009). In addition, sainfoin also has potential for control of parasitic worms (Hoste et al., 2006), for reducing methane (CH_4) emission (McMahon et al., 1999) and nitrogen losses as urine (Scharenberg et al., 2007; Aufrere et al., 2008; Theodoridou et al., 2010) from ruminants. Although it is understood that the condensed tannins (CT) in sainfoin are responsible for these beneficial effects, it is far from clear which particular sainfoin tannins are responsible. A survey of the currently available sainfoin germplasm (Stringano et al., 2012) revealed that (i) sainfoin contains a complex set of different tannin molecules and (ii) different sainfoin accessions vary enormously in their tannin compositions.

A number of studies have screened many plants and plant extracts for their potential to decrease CH_4 production when used as feed additives, as supplementary feeds or as sole feeds for ruminants (Patra et al., 2006; Bodas et al., 2008; Garcia-Gonzalez et al., 2008). These studies have tended to focus on the contents of plant secondary metabolites such as essential oils, saponins or CT. Among these, CT has received much attention for their ability to modify ruminal fermentation patterns. However, CT contents and structures differ substantially not only between plant species but also between cultivars, varieties, accessions, and also between the different plant parts (Lees et al., 1993; Theodoridou et al., 2010; Bodas et al., 2012).

Some previous studies reported variation in CT structures from a few sainfoin accessions (Koupai-Abyazani et al., 1993; Marais et al., 2000). However, a recent evaluation of a large sainfoin germplasm collection from the EU 'HealthyHay' project found substantial variation in tannin sizes (i.e., the mean degree of polymerisation, mDP), monomeric flavanol compositions that give rise to different prodelphinidin:procyanidin ratio (PD:PC) and stereochemistry at the heterocyclic C-ring (*cis:trans* flavanol ratio) and CT contents among accessions (Stringano et al., 2012). This sort of variation could account for some of the contradictory reports on the beneficial effects of sainfoin (reviewed by Mueller-Harvey 2006; Scharenberg et al., 2009; Theodoridou et al., 2011). To our knowledge, such a large CT-

containing forage legume germplasm collection has not yet been evaluated for possible CT structure–activity relationships in terms of ruminal in vitro CH_4 production and fermentation characteristics. The objective of this study was to investigate whether variations in CT structures and contents within a large sainfoin germplasm collection affects rumen in vitro CH_4 production and fermentation characteristics. The aim was to identify promising sainfoin accessions suitable for future investigations and to provide novel guidelines for plant breeders interested in developing new varieties with optimised tannin compositions.

MATERIALS AND METHODS

Forage sample collection and preparation

This study was part of the EU funded 'HealthyHay' project (http://legumeplus.eu/). Sainfoin plants were grown since 2007, and during June and July 2008 a collection of 360 sainfoin accessions were evaluated for their agronomic performances at Cambridge National Institute of Agricultural Botany (NIAB, Cambridge, UK). A subset of 46 accessions was selected for laboratory studies based on morphology, vigour, disease resistance and flower colour (Hayot Carbonero et al., 2011b). The accessions, a collection of plant material from a particular location (Aubry et al., 2005), originated from Central Europe, Eastern Europe, North America and Asia (Table 1). Seeds of each accession were initially tested for germination for two weeks in the greenhouse. Accessions with sufficient germination rate (> 25%) were then sown in the field in May 2007. After initial plantings, up to 30% of the seedlings were found died in the field. Therefore, a second strategy was proposed and used in which the seedlings were grown for longer in the greenhouse (1.5 months instead of two weeks) in big permeable Jiffy pots (Jiffy Products Ltd., UK) using a Rhizobium species inoculum (either the UK1 strain isolated from an *Onobrychis viciifolia* Cotswold Common cultivar or the 6862 USDA strain from the US culture collection) obtained from Legume Technology Ltd., UK. The plants were then transferred individually to the field.

Each accession was sown in rows in replicate plots (plot size = 1.5 m^2, row spacing = 0.25 m) in May 2008. The top layer of the soil was a slightly stony clay loam and the subsoil was characterized by a permeable brown slightly stony clay loam. Sainfoin accessions were harvested when about 50% of stems showed open flowers on the lowest half of the flower stem. All accessions were harvested between 9 June and 1 August 2008. The plants were harvested at 5 cm height above the ground using hand shears. For more detailed information, readers are referred to Hayot Carbonero et al. (2011b). Sample collection and preparation

were done as described previously (Stringano et al., 2012). In brief, plant material was then weighed, packed into Nalgene low-density polyethylene bags and frozen at –20^{o}C. The frozen samples were subsequently transported to the freeze-drying facility at the Archaeological Trust in York, UK. The freeze-dried material was first ground to < 8 mm (knife mill P-15, Retsch, Haan, Germany) and then to < 1 mm using a Wiley mill (T. Peppink and Zn., Machinefabriek, Amsterdam, The Netherlands). Subsequently, the ground material was passed through a sample divider, which generated appropriate sub–samples. Sub–samples were used at Wageningen University (Wageningen, The Netherlands) for the in vitro experiments and had been used at the University of Reading (UK) for the tannin analysis. Sub–samples were stored in a dark and cool (4^{o}C) place until analysis.

Experimental design

All sainfoin accessions were first characterised for their CT structures and contents (Stringano et al., 2012). Then, about 0.5 g (on air DM basis) of finely ground (< 1 mm) freeze-dried sample of each accession was weighed into duplicate bottles (250 ml Schott bottle, Mainz, Germany) per accession within run and with triplicate runs at different time. All accessions (n = 46) were incubated with polyethylene glycol (PEG, MW 6000 Daltons), a tannin-binding agent, at 1:1 (w/w, substrate:PEG) and without PEG. The PEG treatments were referred as +PEG (with PEG) and –PEG (without PEG).

Experimental procedures and sampling

All animal handling procedures were approved of by the Animal Care and Use Committee of Wageningen University and accorded with the Dutch legislation on the use of experimental animals. Cumulative gas production was measured using a fully automated time related gas production system (Cone et al., 1996). Substrates were weighed into 250-ml volume bottles (Schott bottle, Mainz, Germany) and incubated with buffered rumen fluid. Rumen fluid was obtained from three rumen cannulated lactating Holstein−Friesian dairy cows fed grass–maize silage and concentrate based total mixed ration. The cows received a grass and maize silage in the morning and afternoon and 7−8 kg of concentrate (starch = 160 g/kg of DM, CP = 200 g/kg of DM, crude fat = 38 g/kg of DM and ash = 80 g/kg of DM) according to their requirements. Rumen fluid was collected prior to morning feeding and obtained by suction method using a long plastic tube strainer that was inserted into the rumen cannula and creating a vacuum to the other end of the tube connected to a one litre plastic bottle. Rumen fluid was transferred into pre–warmed and carbon dioxide (CO_2)

Table 1. List and origins of sainfoin germplasm accessions[1].

No.	NIAB accession number[2]	Species	Variety	Country of origin
1	1005R2	*O. viciifolia* Scop.	Perly	NA[3]
2	1007R2	*O. viciifolia* Scop.		China
3	1012R2	*O. viciifolia* Scop.	Ambra	Italy
4	1012R1	*O. viciifolia* Scop.	Ambra	Italy
5	1013R2	*O. viciifolia* Scop.	Somborne	NA
6	1017R1	*O. viciifolia* Scop.	Teruel	Spain
7	1017R2	*O. viciifolia* Scop.	Teruel	Spain
8	1019R2	*O. viciifolia* Scop.	Jaja	Poland
9	1026R1	*O. viciifolia* Scop.	Buciansky	Slovakia
10	1026R2	*O. viciifolia* Scop.	Buciansky	Slovakia
11	1028R1	*O. viciifolia* Scop.	Simpro	France
12	1041R2	*O. viciifolia* Scop.	Camaras	Romania
13	1043R2	*O. viciifolia* Scop.	Bivolari	Romania
14	1071R2	*O. viciifolia* Scop.	Hampshire Common	NA
15	1077R2	*O. viciifolia* Scop.	Nova	NA
16	1103R2	*O. viciifolia* Scop.	Korunga	Turkey
17	1104R2	*O. viciifolia* Scop.		Turkey
18	1110R2	*O. viciifolia* Scop.	CPI63750	Turkey
19	1113R2	*O. viciifolia* Scop.	CPI63753	Spain
20	1123R2	*O. viciifolia* Scop.	CPI63763	Turkey
21	1127R2	*O. viciifolia* Scop.	CPI63767	Washington, USA
22	1156R2	*O. viciifolia* Scop.	Dukorastushchii	FSU[4]
23	1157R1	*O. viciifolia* Scop.	Miatiletka	FSU
24	1157R2	*O. viciifolia* Scop.	Miatiletka	FSU
25	1163R2	*O. viciifolia* Scop.	Giant	England
26	1165R1	*O. viciifolia* Scop.	Rees “A”	England
27	1165R2	*O. viciifolia* Scop.	Rees “A”	England
28	1169R2	*O. viciifolia* Scop.	CPI63810	Lithuania
29	1179R1	*O. viciifolia* Scop.	CPI63820	Spain
30	1197R2	*O. viciifolia* Scop.	CPI63838	Norway
31	1199R2	*O. viciifolia* Scop.	CPI63840	FSU
32	1200R2	*O. viciifolia* Scop.	CPI63841	Germany

Table 1. (continued)

No.	NIAB accession number[2]	Species	Variety	Country of origin
33	1210R2	*O. viciifolia* Scop.	Premier	Switzerland
34	1213R2	*O. viciifolia* Scop.	CPI63854	Switzerland
35	1220R2	*O. viciifolia* Scop.	247	Morocco
36	1230R2	*O. viciifolia* Scop.	Visnovsky	Czech Central
37	1253_04R2	*O. viciifolia* Scop.	Tu86-43-04	Turkey, Hakkari
38	1253_03R1	*O. viciifolia* Scop.	Tu86-43-03	Turkey, Hakkari
39	1256R1	*O. viciifolia* Scop.	Wkt 10	Turkey, Afyon
40	1260R2	*O. viciifolia* Scop.	X93234	China, Xinjiang
41	1261R2	*O. viciifolia* Scop.	Line 108	Armenia
42	1261R1	*O. viciifolia* Scop.	Line 107	Armenia
43	1262R1	*O. viciifolia* Scop.	Cotswold Common	Great Britain
44	1262R2	*O. viciifolia* Scop.	Cotswold Common	Great Britain
45	1264R1	*O. antasiatica*	Sisiani local	Armenia
46	1264R2	*O. antasiatica*	Sisiani local	Armenia

[1]List of accessions were published previously in Stringano et al. (2012). Reproduced with permission from the publisher.
[2]R1 and R2 refer to two replicates of the same accession collected from different plots.
[3]NA, not available.
[4]Former Soviet Union.

flushed thermos flasks, transported quickly to the laboratory, pooled and filtered through two layers of cheesecloth into a flask flushed with CO_2.

Rumen fluid was mixed with a buffered mineral solution at 1:2 ratios (v/v) as described by Cone et al. (1996) under constant stirring and continuous flushing with CO_2, while maintained in a water bath (Haake SWB25, Thermo Electron Corporation) at 39^{o}C. Subsequently, 60 ml of buffered rumen fluid was dispensed into pre-warmed fermentation bottles that contains substrate (sainfoin) sample and pre-flushed with CO_2. The bottles were directly incubated at 39^{o}C in a water bath shaking at 40–50 movements per minute. The experiment was repeated during three periods (runs) on different periods (days). During each run, four bottles containing only buffered rumen fluid were included as blanks. Total gas production for each bottle was corrected for the blank.

The buffer–mineral solution contains per litre of 8.75 g $NaHCO_3$, 1.0 g NH_4HCO_3, 1.43 g Na_2HPO_4, 1.55 g KH_2PO_4, 0.15 g $MgSO_4.7H_2O$, 0.017 g $CaCl_2.2H_2O$, 0.015 g $MnCl_2.4H_2O$,

0.002 g $CoCl_2.6H_2O$, 0.012 g $FeCl_3.6H_2O$, 0.3 g $Na_2S.9H_2O$ and 1.25 mg resazurin.

Methane measurement and calculations

Methane concentration in the headspace of the fermentation bottle was measured by gas chromatography (GC; GC8000Top, CE Instruments, Milan, Italy). In order to allow CH_4 sampling from the headspace, fermentation bottles were modified as described by Pellikaan et al. (2011). The bottles were fitted with a glass extension and sealed with a screw cap fitted with an airtight septum (XLB-11 Septa 7/16 GRACE, Breda, The Netherlands). The screw caps were furnished with a small aperture to allow a fine needle to pass. Ten microlitre (10 µl) aliquots of the bottle headspace gas were sampled through this opening at 0, 2, 4, 6, 8, 10, 12, 24, 32 and 48 h of incubation using a gas tight syringe (Gastight® # 1701 Hamilton 1701N, 10 µl Syringe, point style 5, Bonaduz, Switzerland) and directly injected into the GC split injector port. The GC was fitted with a flame ionization detector (FID) and stainless steel column (6 m length, 0.53 mm i.d., 25 µm film thicknesses, and packed with PoraPack Q 50-80 mesh (GRACE, Breda, The Netherlands). The temperatures of the injector, column and detector were maintained at 150, 60 and 150°C, respectively. The carrier gas was nitrogen, and the pressure for nitrogen, hydrogen and air was set at 100, 50 and 100 kPa, respectively.

The CH_4 concentration in the headspace was calculated by external calibration using a certified gas mixture containing a known composition of CH_4 (Linde Gas Benelux, Schiedam, The Netherlands). Peak areas were determined by automatic integration software for GC (Chrom−Card data system Version 2.4, 2006, Rodano Milan, Italy). The methane concentrations were plotted against time and a modified nonlinear monophasic equation (Groot et al., 1996) was fitted through the data points using the nonlinear least squares regression procedure, PROC NLIN of SAS (SAS Institute Inc., 2010). Curve fit parameter estimates or the final estimates of the model parameters describing the increasing CH_4 concentrations in time were then used to compute CH_4 concentrations at each individual valve opening. Cumulative CH_4 production was calculated as the sum of the increase in the amount of CH_4 between two successive valve openings and the amount of CH_4 vented from the bottle using the following formula:

Total methane production = headspace (HS) volume × HS methane concentration + gas production × K × HS methane concentration, where the total gas volume is automatically recorded by the gas production appartus, K is a coefficient and it is the ratio of CH_4 concentration in outflow gas to HS.

The cumulative CH_4 produced was corrected for the initial amount of OM weighed into

individual bottles and reported in ml per gram of OM.

Analytical procedures

Analysis of chemical composition was done at the University of Reading (Reading, UK). Dry matter (DM) and ash were determined using AOAC 967.03 (1995) and AOAC 942.05 (1995), respectively. Neural detergent fibre (NDF) and acid detergent fibre (ADF) were determined using the ANKOM method (ANKOM^{220} Fibre Analyser, Ankom Corp., Fairport, New York, USA). Nitrogen content was determined using Kjeldahl method (ISO 5983, 2005) and CP was calculated by multiplication of total N content by 6.25. The gross energy content of samples was determined in an adiabatic bomb calorimeter (IKA C7000, IKA®-Werke GmbH and Co. KG, Staufen, Germany). Total water-soluble carbohydrates (TWSC) were hydrolysed in dilute sulphuric acid and analysed on an auto analyser system (SFA-2000 instrument, Burkard Scientific, Uxbridge, UK) using the neocuproine/cupric sulphate reagent (Chemlab Instruments Ltd, Great Dunmow, UK).

Volatile fatty acid (VFA) concentration was determined by gas chromatography (GC; Fisons HRGC MEGA2, CI instruments, Milan, Italy) as described by Taweel et al. (2005). Total VFA concentration was corrected for the VFA concentration of blank (i.e. rumen fluid plus buffer) and expressed as mM per gram of OM.

Condensed tannins were analysed by thiolytic degradation as described by Gea et al. (2011). The HPLC data on monomeric flavanol composition in the CT provided information on the mean degree of polymerization (mDP), prodelphinidin:procyanidin (PD:PC) and *cis:trans* ratios for each sainfoin accession (Gea et al., 2011).

$$\text{mDP} = \frac{\text{amount of extension and terminal flavanol units}}{\text{amount of terminal flavanol units}}$$

Prodelphinidin:procyaninidin (PD:PC) ratio of tannins were calculated as:

$$\text{PD:PC} = \frac{\text{percentage of GC + EGC units}}{\text{percentage of C + EC units}}$$

The formula for *cis:trans* ratio was:

$$\text{cis:trans} = \frac{\text{percentage of EC + EGC units}}{\text{percentage of C + GC units}}$$

where C = catechin; EC = epicatechin; GC = gallocatechin; EGC = epigallocatechin.

Statistical analysis

Data per run were averaged before statistical analysis. Repeated measures of total gas and methane production data were analysed using PROC MIXED procedure in SAS with accession, PEG, time, and their interactions (accession × PEG, and accession × PEG × time) as a fixed treatment effects and bottle as a random effect using the repeated measure procedure with time as a repeated variable (timely data obtained from the same bottle as a repeated measure analysis).

Data were analysed according to a 46 × 2 factorial arrangement of treatment (46 accessions × 2 treatments, with or without PEG) using the following model:

$$Y_{ijk} = \mu + A_i + P_j + T_k + (A \times P)_{ij} + (A \times P \times T)_{ijk} + e_{ijk}$$

where Y_{ijk} is the dependant variable, μ is the overall mean, A_i is the effect of accession (I = 46), P_j is the effect of PEG (j = 2), T_k is the effect of time (k = 4); $(A \times P)_{ij}$ is the interaction of accession with PEG, and $(A \times P \times T)_{ijk}$ is interactions of accession with PEG and with time; e_{ijk} is the residual error term. Data for VFA and fermentation kinetics parameter estimates (B1 and half time) were based on end time point measurement. Therefore, the model does not include time as a repeated variable for these variables, and data were subjected to ANOVA using the PROC GLM procedure in SAS. Post hoc analysis were performed using the Tukey-Kramer multiple range tests for pairwise comparison with least square means declared significant at a probability of $P \leq 0.05$. Tendency was declared at $0.05 < P < 0.10$.

To understand the major patterns of variations among the sainfoin accessions and establish correlation among multiple traits (nutritional composition, tannin structure and CH_4 production) principal component analysis (PCA) was performed using the program CANOCO version 4.5 (Ter Braak and Šmilauer, 2002). To reduce the dimensionality of the dataset, PCA was based on optimally weighted observed variables. The data was log-transformed to ensure normality and the correlation matrix was used for the calculation after the data were mean-centred and standardized. The transformed new variables referred to as principal components (PCs) are the linear combinations of the original variables. Each PC is independent and orthogonal to each other. By plotting the PCs, the interrelationships between each accession in terms of nutritional or tannin characteristics and fermentation products were shown in a two dimensional PCA correlation biplots. In the correlation biplots, variables with high positive correlations have acute angles between their vectors. The length indicates the strength of the variable in relation to the displayed ordination (Ter Braak, 1994). The direction of each

variable in the loading plot describes its relationship to the other variables and the position of individual accession on the plot describes their classification along the two principal components.

RESULTS

Nutritional composition and condensed tannins characteristics

The nutritional composition and CT characteristics of sainfoin accessions are shown in Table 2. Large variations were observed among the accessions. Total water-soluble carbohyd-

Table 2. Forage composition (g/kg of DM, unless otherwise stated), condensed tannins (CT) content and CT composition of selected sainfoin accessions[1].

NIAB accession number[2]	Chemical composition						CT content and composition[3]			
	DM (g/kg)	Ash	TWSC	ADF	NDF	CP	CT	mDP	PD	*trans*
1005R2	929.0	60.5	100.7	333.9	496.6	128.0	12.5	26.4	70.5	24.5
1007R2	917.4	44.5	124.5	364.7	508.6	91.0	11.4	35.0	81.8	23.1
1012R1	911.9	61.1	83.4	281.0	423.3	161.0	12.9	27.4	75.5	19.5
1012R2	931.0	50.4	74.2	407.0	560.8	106.0	8.7	30.9	79.3	25.6
1013R2	925.8	56.8	118.5	316.8	493.1	117.0	15.3	29.8	71.0	27.3
1017R1	919.0	52.0	99.7	375.9	510.0	112.0	9.1	27.2	72.3	24.2
1017R2	919.9	60.4	84.2	292.9	499.1	147.0	12.9	29.6	71.4	17.6
1019R2	917.5	51.0	142.8	313.6	476.6	90.0	14.6	21.6	77.9	23.6
1026R1	923.4	56.9	86.7	349.5	488.5	119.0	9.3	23.0	75.6	28.6
1026R2	918.4	60.8	101.5	295.2	499.8	128.0	13.6	27.3	80.2	26.5
1028R1	926.8	55.4	78.4	350.5	487.4	141.0	7.9	32.6	72.0	21.3
1041R2	921.3	38.4	116.9	346.4	550.1	77.0	11.6	38.4	79.1	14.9
1043R2	937.8	48.0	114.2	308.5	503.2	118.0	12.9	21.3	84.2	22.5
1071R2	938.0	59.6	82.5	284.8	453.9	142.0	10.5	12.0	67.8	20.5
1077R2	936.9	48.6	74.2	444.8	571.3	109.0	7.1	74.3	78.7	12.0
1103R2	938.0	42.7	105.1	358.3	511.7	112.0	9.0	18.2	78.8	20.8
1104R2	931.1	49.1	77.7	357.2	507.3	110.0	7.0	49.5	78.9	30.8
1110R2	923.7	55.6	79.9	339.7	496.6	129.0	7.8	20.7	80.5	31.3
1113R2	929.8	58.9	93.4	361.1	500.6	116.0	8.2	82.6	78.6	25.9
1123R2	926.4	47.9	79.3	366.7	504.2	116.0	8.8	20.4	80.6	29.9
1127R2	930.6	51.3	80.7	366.2	517.9	113.0	9.0	84.0	82.8	29.6

Table 2. (continued)

accession number	DM (g/kg)	Ash	TWSC	ADF	NDF	CP	CT	mDP	PD	*trans*
1156R2	923.6	55.1	90.9	336.2	558.2	127.0	8.5	19.4	78.6	34.0
1157R1	923.3	43.2	90.0	343.3	469.6	124.0	9.6	26.5	78.0	26.9
1157R2	924.6	51.3	104.1	343.4	561.1	125.0	11.6	32.8	80.9	28.5
1163R2	937.0	55.9	88.5	388.4	509.9	117.0	12.4	27.8	64.8	23.7
1165R1	920.5	56.0	121.0	242.3	452.2	166.0	15.6	19.4	65.7	23.5
1165R2	917.5	52.6	113.8	248.0	365.3	141.0	20.1	21.8	67.6	19.6
1169R2	923.6	49.6	122.1	243.3	490.6	148.0	20.1	29.7	77.9	18.8
1179R1	932.7	57.8	95.6	362.6	584.9	142.0	11.0	17.9	52.7	16.8
1197R2	932.1	52.2	82.9	357.3	550.4	118.0	9.0	32.9	80.4	23.8
1199R2	920.6	55.4	73.0	392.6	529.2	122.0	8.9	28.1	77.9	19.8
1200R2	909.7	58.1	99.3	282.6	403.0	162.0	9.1	18.5	72.9	18.7
1210R2	917.1	55.3	113.3	267.1	460.6	166.0	11.7	24.1	72.6	20.8
1213R2	909.4	65.3	96.2	260.4	387.5	144.0	17.3	27.4	80.1	19.0
1220R2	885.3	57.7	128.5	235.1	337.7	159.0	6.4	24.9	83.1	27.2
1230R2	934.0	48.9	121.6	309.2	503.2	125.0	10.7	15.8	80.8	24.8
1253_03R1	936.1	48.5	77.9	352.0	550.1	123.0	9.2	15.7	85.2	22.2
1253_04R2	914.8	57.1	80.3	383.1	517.0	123.0	10.0	14.7	85.1	23.3
1256R1	942.3	49.8	47.9	377.4	559.8	100.0	28.0	17.6	94.8	31.6
1260R2	928.8	53.8	67.4	397.4	529.7	120.0	8.0	26.4	83.9	26.3
1261R1	914.3	59.0	84.3	287.1	411.4	151.0	7.1	46.1	79.4	18.8
1261R2	952.3	49.8	77.2	411.4	553.3	108.0	9.7	44.8	74.3	23.2
1262R1	935.9	52.5	84.6	315.8	448.9	149.0	12.0	25.8	67.8	20.4
1262R2	916.6	65.3	84.0	291.0	432.1	144.0	15.7	38.1	66.7	20.0
1264R1	916.4	53.5	102.8	295.5	411.6	148.0	10.0	60.0	78.3	21.5
1264R2	924.8	53.2	96.2	354.6	490.0	125.0	5.7	58.5	80.9	21.9
Mean	924.9	53.6	94.4	332.4	491.9	127.0	11.3	31.5	76.7	23.4
Minimum	885.3	38.4	47.9	235.1	337.7	77.0	5.7	12.0	52.7	12.0
Maximum	952.3	65.3	142.8	444.8	584.9	166.0	28.0	84.0	94.8	34.0
S.D.	11.0	5.7	19.0	49.5	55.8	20.5	4.1	16.8	7.0	4.6

[1]Results for mDP, percentage PD and percentage trans were published previously as Supplementary Info (Table S1) in Stringano et al. (2012). Reproduced with permission from the publisher.

[2]R1 and R2 refer to two replicate samples of the same accession collected from different plots.

[3]CT, Condensed tannins (g/kg of freeze-dried sainfoin plant material); mDP, mean degree of

polymerisation; PD, mean proportion of prodelphinidin in tannin molecules (% PD = 100 – mean proportion of PC); *trans*, mean proportion of *trans* flavanol units in tannin molecules (% *trans* = 100 – mean proportion of *cis*).

rates (TWSC) showed a 3.0-fold variation (range from 47.9 to 142.8 g/kg of DM) followed by a 2.2-fold variation for CP (range from 77 to 166 g/kg of DM), ADF varied from 235.1 to 444.8 g/kg of DM and NDF from 337.7 to 584.9 g/kg of DM. Dry matter varied least from 885.3 to 952.3 g/kg).

Condensed tannins concentrations ranged from 5.7 (accession 1264R2) to 28.0 g per kilogram of freeze-dried sainfoin plant material (accession 1256R1). There was also a considerable variation in CT composition as reported previously (Stringano et al., 2012). This variation gave rise to prodelphinidin:procyanidin (PD:PC) ratio that ranged from 52.7:47.3 to 94.8:5.2 (accessions 1179R1 and 1256R1, respectively); to mean degrees of polymerisation (mDP) that ranged from 12.0 in accession 1071R2 to 84.0 in accession 1127R2; and the proportion of *trans* varied from 12.0 to 34.0 in accessions 1077R2 and 1156R2, respectively.

In vitro gas and methane production

With respect to the total dataset of the 46 sainfoin accessions, +PEG on average increased total gas (GP) by 7.8 ml/g of OM at 6 h of incubation, which equals to a 5.6 % increase relative to the GP produced by sainfoin –PEG treatment (Table 3). Similarly, the +PEG compared to –PEG treatment increased GP by 4.1% and 2.8% at 12 and 24 h. The highest increase in GP as a result of +PEG treatment was 17.0% and 11.0% (accession 1165R2 at 6 h and accession 1123R2 at 24 and 48 h, respectively), but addition of PEG has no statistical significant effect on GP at different time points (Table 3).

Methane production differed among accessions and was affected by time of incubation ($P < 0.001$; Table 4). Addition of PEG has significant effects on CH_4 production. The average cumulative CH_4 production with –PEG treatment was 17.1 ± 4.2 ml/g of OM (10.9 to 27.9 ml/g of OM), 29.7 ± 4.5 ml/g of OM (19.8 to 39.4 ml/g of OM), 43.3 ± 4.8 ml/g of OM (31.3 to 53.6 ml/g of OM) and 53.5 ± 5.7 ml/g of OM (42.6 to 66.5 ml/g of OM) at 6, 12, 24 and 48 h, respectively. Addition of PEG resulted in a general but small increase in CH_4 production for most accessions (average increases of 11.1, 9.2, 6.8 and 5.0% were observed at 6, 12, 24 and 48 h, respectively). However, the interaction between accession and PEG showed only a tendency ($P = 0.080$) to affect CH_4 (Table 4), while GP was not affected (Table 3). The CH_4 produced after 24 h averaged 18.7% and 19.5% of total gas with and without PEG.

Table 3. In vitro gas production and fermentation kinetics of sainfoin accessions incubated with (+PEG) and without polyethylene glycol (−PEG).

NIAB accession (A) number[1]	Total gas (ml/g of incubated OM)								Kinetics parameter estimates			
	6 h		12 h		24 h		48 h		B1		Half-time (h)	
	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG
1005R2	145.1	153.0	194.3	201.2	235.4	240.8	264.1	268.4	0.95	0.95	6.6	6.0
1007R2	144.2	149.3	193.0	197.5	235.9	241.2	268.2	275.4	0.87	0.80	7.5	7.8
1012R1	155.2	163.9	207.3	214.7	250.6	256.0	280.8	284.4	0.95	0.95	6.5	5.8
1012R2	127.5	135.0	171.8	180.6	213.3	222.6	246.8	255.8	0.80	0.87	9.1	8.5
1013R2	146.9	165.6	196.2	215.0	238.3	254.2	268.7	280.8	0.90	0.95	6.9	5.5
1017R1	137.6	148.5	183.4	193.8	223.7	232.8	253.8	261.7	0.90	0.85	7.3	6.6
1017R2	148.9	154.7	198.6	203.3	239.5	242.0	267.5	268.0	1.00	1.00	6.3	5.7
1019R2	148.7	158.7	195.7	205.7	233.4	242.9	259.1	268.0	0.95	0.95	5.9	5.4
1026R1	133.2	146.7	178.3	193.6	217.1	232.0	245.3	258.4	0.90	0.95	7.0	6.1
1026R2	142.3	148.1	188.1	192.7	225.3	227.3	250.7	250.0	0.95	1.00	6.1	5.3
1028R1	126.7	138.7	169.8	184.8	206.4	222.3	232.4	247.9	0.97	0.97	6.8	6.2
1041R2	130.9	135.4	176.7	179.7	216.9	215.5	246.7	239.9	0.93	0.95	7.6	6.1
1043R2	132.0	149.3	177.9	196.1	216.7	232.6	244.2	256.5	0.95	1.00	6.9	5.6
1071R2	138.0	144.1	187.1	191.9	228.9	230.5	258.6	256.5	1.00	1.00	7.1	6.1
1077R2	109.9	115.0	149.3	155.6	186.4	193.0	216.3	222.2	0.85	0.90	9.4	8.6
1103R2	133.7	139.6	180.0	185.1	220.5	222.7	250.5	248.8	0.90	0.95	7.5	6.3
1104R2	152.0	157.6	202.3	205.8	248.4	249.8	284.7	285.1	0.85	0.80	8.4	8.3
1110R2	145.9	140.8	195.8	188.9	241.6	231.2	277.8	262.8	0.87	0.90	8.6	7.5

Table 3. (continued)

NIAB accession (A) number[1]	Total gas (ml/g of incubated OM)								Kinetics parameter estimates			
	6 h		12 h		24 h		48 h		B1		Half-time (h)	
	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG
1113R2	148.2	153.0	196.6	199.8	241.1	242.4	276.7	276.4	0.85	0.80	8.6	8.1
1123R2	125.7	140.4	168.8	187.3	208.6	230.7	240.0	265.6	0.80	0.83	8.6	9.0
1127R2	135.4	145.5	182.4	192.5	226.3	234.6	261.8	267.1	0.83	0.87	9.4	7.7
1156R2	149.5	152.4	198.7	200.9	248.1	244.6	292.0	278.9	0.70	0.85	12.2	8.0
1157R1	142.5	149.3	190.8	198.5	235.8	243.1	271.8	278.2	0.85	0.85	9.1	8.5
1157R2	139.6	145.8	187.4	193.9	232.5	238.8	269.2	275.2	0.85	0.80	9.6	9.2
1163R2	124.0	139.0	168.3	184.9	212.0	228.0	249.6	263.1	0.80	0.80	11.8	9.4
1165R1	153.4	165.6	204.5	217.3	248.7	260.1	281.4	290.6	0.90	0.93	7.2	6.4
1165R2	147.8	173.4	198.4	219.3	243.5	259.1	277.8	289.6	0.90	0.80	8.0	6.0
1169R2	157.2	169.7	212.1	223.2	264.0	272.9	306.2	313.1	0.80	0.80	9.5	8.4
1179R1	105.3	115.3	148.9	158.6	191.3	199.5	225.9	232.4	0.90	0.90	11.2	9.8
1197R2	138.8	119.9	187.9	162.8	237.9	203.6	282.6	236.7	0.70	0.87	13.2	9.8
1199R2	131.6	135.4	180.4	183.5	232.1	234.1	280.5	281.3	0.70	0.70	16.5	16.1
1200R2	144.1	161.2	195.3	213.4	245.3	263.6	287.5	305.7	0.80	0.80	10.7	9.8
1210R2	166.4	169.3	220.7	221.4	265.8	262.1	297.6	288.7	0.95	1.00	6.5	5.5
1213R2	144.8	151.9	196.5	203.9	240.0	247.1	270.7	277.0	0.97	0.95	7.1	6.6
1220R2	166.9	172.4	219.6	223.8	262.6	266.1	292.3	295.8	0.95	0.90	6.0	5.9
1230R2	142.8	161.2	196.0	213.5	242.3	257.0	275.6	287.4	0.95	0.93	7.7	6.4

Table 3. (continued)

NIAB accession (A) number[1]	Total gas (ml/g of incubated OM)								Kinetics parameter estimates			
	6 h		12 h		24 h		48 h		B1		Half-time (h)	
	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG
1253_03R1	134.4	142.7	182.4	190.8	225.5	232.4	258.1	263.1	0.90	0.90	8.2	7.4
1253_04R2	136.0	150.9	185.9	200.2	229.5	242.4	261.3	273.0	0.95	0.90	7.7	6.9
1256R1	141.5	152.7	190.5	202.7	229.7	241.3	255.6	265.9	1.05	1.05	6.2	5.7
1260R2	139.7	131.6	177.5	177.8	211.4	218.8	237.6	249.5	0.80	0.90	6.6	7.9
1261R1	154.4	150.0	206.1	201.6	249.0	243.7	279.0	272.2	0.95	1.00	6.5	6.5
1261R2	127.2	135.0	172.3	181.7	214.0	222.5	246.8	252.5	0.90	0.90	8.9	7.6
1262R1	143.8	148.3	191.7	198.4	231.5	238.9	259.1	266.1	1.00	1.00	6.5	6.3
1262R2	137.1	150.3	185.4	198.6	226.2	237.8	255.2	264.4	0.97	0.93	7.1	6.0
1264R1	159.6	155.3	208.9	206.5	249.0	249.3	276.6	279.5	0.90	0.95	5.8	6.6
1264R2	135.7	150.1	183.6	200.1	227.1	242.8	260.7	273.5	0.90	0.95	8.4	6.8

	SEM[2]	*P*-value[3]
A	4.48	<0.001
PEG	0.93	<0.001
Time	1.87	<0.001
A × PEG	6.34	0.750
A × PEG × Time	12.68	1.000

	P-value[4]	*P*-value[4]
A	<0.001	<0.001
PEG	0.272	<0.001
Time	–	–
A × PEG	1.000	1.000

[1]R1 and R2 refer to two replicate samples of the same accessions collected from different plots.

[2]SEM, standard error of the means.

[3]*P*-value for total gas.

[4]*P*-value for kinetics parameter estimates. Data for fermentation kinetics parameter estimates (B1 and half time) were based on end time point measurement. Therefore, the model does not include time as a repeated variable.

Table 4. In vitro methane production and fermentation kinetics of sainfoin accessions incubated with (+PEG) and without polyethylene glycol (−PEG).

NIAB accession (A) number	Methane production (ml/g of incubated OM)								Kinetics parameter estimates			
	6 h		12 h		24 h		48 h		B1		Half-time (h)	
	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG
1005R2	21.5	25.1	38.2	37.7	50.9	50.0	57.8	59.4	1.65	1.00	9.7	11.6
1007R2	17.1	17.0	32.4	31.3	46.9	45.8	55.9	55.6	1.57	1.40	12.3	12.3
1012R1	20.0	20.5	35.1	34.7	49.4	49.8	58.7	61.8	1.35	1.20	11.4	15.1
1012R2	12.9	17.9	26.8	32.9	40.1	47.3	47.2	56.7	1.70	1.43	11.3	12.6
1013R2	18.9	19.2	32.8	34.1	47.0	48.6	57.4	57.9	1.30	1.40	14.3	11.3
1017R1	18.4	19.1	31.7	33.4	45.3	47.8	55.6	57.8	1.25	1.35	15.4	12.2
1017R2	19.1	18.8	32.7	33.5	46.4	48.5	56.2	58.8	1.25	1.35	12.3	12.8
1019R2	16.4	19.6	26.5	36.1	38.3	49.5	49.2	56.4	1.05	1.65	19.2	19.7
1026R1	17.1	18.0	29.3	34.6	42.0	47.8	51.5	54.9	1.20	1.65	13.3	11.0
1026R2	16.2	17.3	28.4	31.4	41.1	45.2	50.4	54.3	1.30	1.40	13.0	12.9
1028R1	15.5	16.9	29.7	30.9	42.9	44.2	50.8	52.5	1.53	1.47	12.0	11.6
1041R2	13.3	15.6	26.9	30.6	41.2	44.0	50.3	51.0	1.57	1.65	12.8	10.7
1043R2	15.6	16.4	28.2	31.2	42.0	44.4	52.5	51.5	1.25	1.60	14.7	10.6
1071R2	14.9	16.4	28.0	31.1	42.5	46.4	53.2	56.8	1.35	1.40	14.6	13.5
1077R2	11.2	13.9	24.3	27.1	38.2	40.6	46.4	49.2	1.70	1.50	13.4	12.5
1103R2	13.3	15.0	26.0	29.6	41.1	43.8	51.9	52.6	1.45	1.55	15.2	13.0
1104R2	22.7	35.2	35.3	46.8	49.6	59.0	63.0	70.1	0.95	0.70	13.9	13.4
1110R2	20.6	16.8	33.9	29.7	48.6	43.4	60.9	53.6	1.10	1.30	16.0	13.4

Table 4. (continued)

NIAB accession (A) number	Methane production (ml/g of incubated OM)								Kinetics parameter estimates			
	6 h		12 h		24 h		48 h		B1		Half-time (h)	
	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG
1113R2	17.5	19.6	29.7	29.9	44.3	41.2	57.7	51.4	1.10	0.90	19.1	22.4
1123R2	17.0	21.5	29.2	33.4	41.0	46.3	48.9	57.6	1.30	1.00	11.3	18.0
1127R2	20.7	23.5	31.3	34.5	42.6	46.0	52.0	55.6	0.97	0.93	14.6	13.7
1156R2	22.6	44.0	35.8	59.6	50.7	75.5	64.1	89.3	1.00	0.85	17.1	12.9
1157R1	15.8	28.7	24.1	40.3	33.9	52.6	43.5	63.3	0.80	0.85	16.7	14.9
1157R2	19.3	21.5	32.2	33.9	47.0	48.2	60.3	61.6	1.10	0.95	17.6	20.9
1163R2	24.6	27.0	34.0	35.6	44.3	44.7	54.0	53.1	0.75	0.65	18.3	14.3
1165R1	19.9	19.9	30.0	32.7	40.8	46.6	49.7	58.3	0.97	1.10	15.8	16.0
1165R2	19.2	18.6	31.7	30.6	46.5	45.8	60.1	61.4	1.05	1.05	19.5	15.9
1169R2	27.9	30.1	38.9	41.8	50.9	54.0	62.0	64.5	0.75	0.85	17.3	13.5
1179R1	22.1	18.3	33.7	29.4	46.1	42.3	56.6	54.6	0.95	0.95	14.7	19.8
1197R2	21.3	23.8	33.9	34.1	47.8	45.4	59.7	55.5	1.00	0.87	15.7	16.1
1199R2	26.4	25.3	39.4	36.3	53.6	48.4	66.5	59.5	0.90	0.85	16.4	17.1
1200R2	20.9	18.1	34.6	30.2	50.0	45.6	63.0	61.0	1.10	1.05	16.0	13.5
1210R2	15.9	14.9	28.5	28.8	42.5	44.3	52.2	54.8	1.47	1.50	13.5	13.9
1213R2	14.0	17.8	27.9	30.4	41.6	44.1	49.8	55.0	1.65	1.20	13.4	14.4
1220R2	15.1	15.2	31.3	33.6	46.7	48.4	55.1	55.8	1.73	1.97	13.1	12.6
1230R2	18.3	15.7	30.7	32.7	44.0	47.9	54.7	55.7	1.15	1.78	14.3	11.6

Table 4. (continued)

NIAB accession (A) number	Methane production (ml/g of incubated OM)								Kinetics parameter estimates			
	6 h		12 h		24 h		48 h		B1		Half-time (h)	
	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG
1253_03R1	13.1	13.7	25.5	26.8	39.4	41.3	49.1	50.9	1.50	1.57	15.3	14.8
1253_04R2	13.2	13.8	26.0	27.4	40.3	42.2	49.7	51.7	1.57	1.57	14.7	13.7
1256R1	13.2	13.1	24.5	26.0	37.5	40.3	47.2	50.1	1.43	1.53	16.6	14.6
1260R2	11.0	13.3	19.8	26.1	31.3	39.5	42.6	48.4	1.20	1.55	14.4	14.7
1261R1	13.0	13.3	25.4	27.3	40.1	42.6	50.7	52.5	1.50	1.63	15.3	14.0
1261R2	12.7	13.2	24.1	27.4	37.3	40.6	46.8	47.7	1.50	1.80	16.5	12.3
1262R1	12.7	13.2	25.7	27.1	39.3	41.3	47.9	49.9	1.63	1.70	16.2	13.0
1262R2	11.7	13.6	24.0	27.4	38.7	42.0	48.6	51.1	1.63	1.63	15.0	13.5
1264R1	10.9	13.3	24.0	27.2	40.3	43.3	50.6	53.8	1.80	1.63	14.7	14.5
1264R2	12.8	12.0	24.8	25.9	39.1	40.6	49.8	49.3	1.43	1.73	16.0	13.2

	SEM[2]	*P*-value[3]
A	1.85	<0.001
PEG	0.39	<0.001
Time	0.55	<0.001
A × PEG	2.62	0.080
A × PEG × Time	5.23	1.000

	P-value[4]	
A	<0.001	0.038
PEG	0.661	0.110
Time	–	–
A × PEG	1.000	0.185

[1]R1 and R2 refer to replicate samples of the same accession collected from different plots; [2]SEM, SE of the means.
[3]*P*-value for total gas.[4]*P*-value for kinetics parameter estimates. Data for fermentation kinetics parameter estimates (B1 and half time) was based on end time point measurement. Therefore, the model does not include time as a repeated variable.

Table 5. Volatile fatty acids production of sainfoin accessions incubated with (+PEG) and without polyethylene glycol (−PEG).

NIAB accession (A) number[1]	Total VFA (mM per OM)		Molar proportion (% of total VFA concentration)											
			Acetate (C2)		Propionate (C3)		Butyrate (C4)		Valerate (C5)		Total iso-acids		C2:C3	
	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG
1005R2	6.5	6.7	65.7	64.3	18.5	19.6	10.0	9.9	1.7	1.9	4.3	4.5	3.5	3.3
1007R2	6.4	6.8	65.7	65.6	18.4	18.8	10.4	9.9	1.6	1.7	4.1	4.0	3.6	3.5
1012R1	6.8	6.4	65.6	65.0	18.7	19.3	9.7	9.3	1.8	1.9	4.5	4.5	3.5	3.4
1012R2	5.7	6.0	66.2	65.1	17.5	19.1	10.3	9.7	1.8	1.8	4.6	4.3	3.8	3.4
1013R2	6.2	6.2	65.2	64.2	18.7	19.6	10.4	10.2	1.7	1.8	4.3	4.3	3.5	3.3
1017R1	5.9	6.5	65.3	65.0	18.6	19.1	10.2	9.8	1.7	1.8	4.4	4.3	3.5	3.4
1017R2	6.2	6.5	64.9	64.8	18.7	19.1	10.3	9.9	1.8	1.8	4.7	4.4	3.5	3.4
1019R2	6.1	6.3	64.5	64.2	18.7	19.2	11.1	10.8	1.7	1.7	4.2	4.1	3.5	3.3
1026R1	6.0	6.0	64.5	63.6	19.1	19.5	10.3	10.4	1.8	1.9	4.6	4.6	3.4	3.3
1026R2	6.5	6.0	66.0	62.7	17.8	19.5	10.4	10.9	1.7	2.0	4.4	4.9	3.7	3.2
1028R1	6.4	6.6	65.5	65.0	18.4	18.6	10.0	9.9	1.8	1.9	4.6	4.6	3.6	3.5
1041R2	5.9	5.8	65.1	64.5	18.3	18.7	10.9	10.8	1.7	1.8	4.3	4.2	3.6	3.4
1043R2	6.0	6.0	64.8	63.3	18.4	19.4	11.0	10.9	1.8	1.9	4.5	4.6	3.5	3.3
1071R2	5.5	6.4	64.6	64.0	18.8	19.5	10.2	9.9	1.8	1.9	4.8	4.7	3.4	3.3
1077R2	5.6	6.4	64.9	64.3	18.5	19.1	10.3	10.0	1.8	1.9	4.7	4.6	3.5	3.4
1103R2	5.9	6.0	64.8	64.0	18.7	19.3	10.7	10.4	1.8	1.9	4.5	4.4	3.5	3.3
1104R2	7.4	7.4	66.3	65.6	18.3	18.8	9.9	9.7	1.7	1.9	4.2	4.0	3.6	3.5
1110R2	7.5	7.2	65.9	66.5	18.8	18.9	9.7	9.2	1.7	1.3	4.3	4.4	3.5	3.0

Table 5. (continued)

NIAB accession (A) number[1]	Total VFA (mM per OM)		Molar proportion (% of total VFA concentration)											
			Acetate (C2)		Propionate (C3)		Butyrate (C4)		Valerate (C5)		Total iso-acids		C2:C3	
	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG
1113R2	7.4	7.5	66.4	65.7	18.1	18.6	9.8	9.8	1.7	1.8	4.2	4.1	3.7	3.5
1123R2	7.0	7.3	66.8	65.6	17.2	18.7	10.1	9.7	1.9	2.0	4.6	4.0	3.9	3.5
1127R2	7.1	7.2	65.7	65.6	18.6	18.7	10.0	9.8	1.8	1.9	4.4	4.0	3.5	3.5
1156R2	7.4	7.4	65.6	65.3	18.8	19.0	9.9	9.7	1.8	1.9	4.4	4.1	3.5	3.4
1157R1	7.1	7.3	65.5	65.3	18.6	18.9	10.0	9.7	1.8	1.9	4.5	4.2	3.5	3.5
1157R2	7.1	7.9	65.2	64.9	18.7	18.9	10.3	10.2	1.7	1.8	4.4	4.2	3.5	3.4
1163R2	7.6	7.0	65.2	65.0	18.9	18.8	10.2	10.1	1.7	1.8	4.3	4.2	3.5	3.4
1165R1	8.5	7.5	66.1	63.9	18.6	19.9	9.5	9.7	1.9	2.1	4.5	4.5	3.6	3.2
1165R2	7.4	7.3	66.1	63.8	18.4	19.5	9.8	10.2	1.7	1.9	4.3	4.6	3.6	3.3
1169R2	7.5	7.6	65.6	64.8	19.0	19.6	9.7	9.5	1.8	1.9	4.3	4.1	3.5	3.3
1179R1	7.6	7.8	65.2	64.7	18.8	19.1	9.9	9.9	1.8	1.9	4.6	4.5	3.5	3.4
1197R2	6.3	6.8	67.1	64.2	15.5	19.0	11.0	10.6	1.9	1.9	4.9	4.4	4.3	3.4
1199R2	7.6	7.3	65.9	65.7	18.5	18.7	9.8	9.6	1.8	2.0	4.4	4.1	3.6	3.5
1200R2	6.7	7.8	65.8	65.2	18.6	19.0	9.7	9.6	1.8	1.9	4.5	4.3	3.5	3.4
1210R2	7.7	8.0	64.0	62.8	19.1	20.2	10.9	10.6	1.9	2.0	4.6	4.4	3.3	3.1
1213R2	8.2	7.9	64.3	63.7	19.0	19.6	10.8	10.5	1.8	1.9	4.5	4.3	3.4	3.3
1220R2	8.0	8.2	63.9	63.8	18.9	19.3	11.0	10.6	2.0	2.0	4.8	4.3	3.4	3.3
1230R2	7.5	7.9	63.9	63.5	19.0	19.5	11.4	11.0	1.8	1.9	4.3	4.1	3.4	3.3

Table 5. (continued)

NIAB accession (A) number[1]	Total VFA (mM per OM)		Molar proportion (% of total VFA concentration)										C2:C3	
			Acetate (C2)		Propionate (C3)		Butyrate (C4)		Valerate (C5)		Total iso-acids			
	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG	−PEG	+PEG
1253_03R1	8.3	7.7	64.4	63.5	18.8	19.6	10.8	10.5	1.9	2.0	4.6	4.3	3.4	3.2
1253_03R2	7.5	8.4	64.5	64.1	18.7	19.2	10.9	10.5	1.8	1.9	4.6	4.2	3.4	3.3
1256R1	7.6	7.8	64.9	63.9	19.0	19.6	10.3	10.2	1.8	2.0	4.4	4.3	3.4	3.3
1260R2	6.7	7.4	62.4	63.9	20.0	19.2	11.3	10.7	2.0	1.9	4.9	4.3	3.1	3.3
1261R1	8.5	8.1	64.1	64.0	19.3	19.3	10.6	10.5	1.9	1.9	4.6	4.3	3.3	3.3
1261R2	7.3	7.2	64.5	64.1	18.9	19.1	10.7	10.6	1.9	2.0	4.5	4.1	3.4	3.4
1262R1	7.6	7.8	64.5	64.1	18.9	19.1	10.8	10.6	1.8	1.9	4.5	4.2	3.4	3.4
1262R2	7.9	7.9	64.9	64.0	18.9	19.6	10.4	10.2	1.8	1.9	4.5	4.3	3.4	3.3
1264R1	7.6	7.9	64.2	63.7	18.8	19.3	11.0	10.8	1.9	2.0	4.7	4.3	3.4	3.3
1264R2	7.5	7.4	64.1	63.9	18.6	18.9	11.3	11.0	1.9	1.9	4.6	4.2	3.5	3.4
						SEM[2]								
A	0.03		0.41		0.01		0.10		0.06		1.93		0.03	
PEG	0.01		0.08		0.01		0.02		0.01		0.40		0.01	
A × PEG	0.04		0.58		0.02		0.14		0.09		2.73		0.04	
						P-value								
A	< 0.001		< 0.001		< 0.001		< 0.001		< 0.001		< 0.001		< 0.001	
PEG	< 0.001		< 0.001		< 0.001		< 0.001		< 0.001		< 0.001		< 0.001	
A × PEG	< 0.001		< 0.001		< 0.001		< 0.001		< 0.001		< 0.001		< 0.001	

[1]R1 and R2 refer to two replicate samples of the same accession collected from different plots; [2]SEM, SE of the means.
Data for VFA was based on end time point measurement. Therefore, the model does not include time as a repeated variable.

The half-time parameters of fermentation kinetics were affected by accession and PEG for GP ($P < 0.001$; Table 3) and only by accession ($P = 0.038$) for CH_4 (Table 4). The highest half time for GP was observed with accession 1199R2 for both +PEG and –PEG (16.1 and 16.5 h, respectively) treatments. Accession 1264R1 yielded the lowest half time (5.8 h) without PEG and accession 1026R2 (5.3 h) with PEG (Table 3). Without PEG, accession 1005R2 required the least time to produce 50% of its maximum CH_4 (Table 4). However, taken together there was no accession × PEG interaction effect on GP (Table 3) and CH_4 (Table 4) half times.

Correlation among nutritional composition, CT structure, in vitro CH_4 production and other fermentation products

Figure 1 presents the principal component analysis (PCA) ordination as a two-dimensional correlation biplots. PCA analysis was made based on the fermentation product data without PEG (−PEG). The first two principal component axes, PC1 (57.6%) and PC2 (18.4%), explained 76.0% of the total variation in the data. Interestingly, there was significant correlation among tannin composition and nutritional parameters with CH_4 production in some sainfoin accessions. Four groups of variables with positive or negative correlations could be identified. Group I consists of variables that include nutritional (NDF, ADF) and CT composition (PD:PC) parameters. Group II explains the CP content of the accessions. Group III describes contributions by mDP, TWSC and the C2:C3 ratio, while Group IV comprises GP, CH_4, TVFA, C2, C3, C4, and total iso-acids data. Based on the dispersion of the accessions on the loading plot, several groups could be identified. Sainfoin accession represented by number 4, 6, 10, 13, 16, 20 and 46 (Figure 1) fell into group I. There was a strong negative correlation of fibre (NDF and ADF) with GP and CH_4 production for this group. The PD:PC ratio was the most important factor among the CT properties considered and showed a negative correlation with the total gas, CH_4 and total VFA produced for accessions in group I.

The CP content tended to show a positive association with fermentation characteristics such as total iso-acids. On the other hand, the TWSC showed a weak relationship with gas production characteristics, although there was a strong positive correlation with the C2:C3 ratio. The dietary CP content was negatively related to mDP, TWSC and C2:C3 ratio. Accessions numbered 24, 32 and 42 were close to the origin and, therefore, cannot reliably be described as falling into this group. The *trans:cis* ratio was associated with the neutral axis and this might suggest that no correlation (either positive or negative) exists.

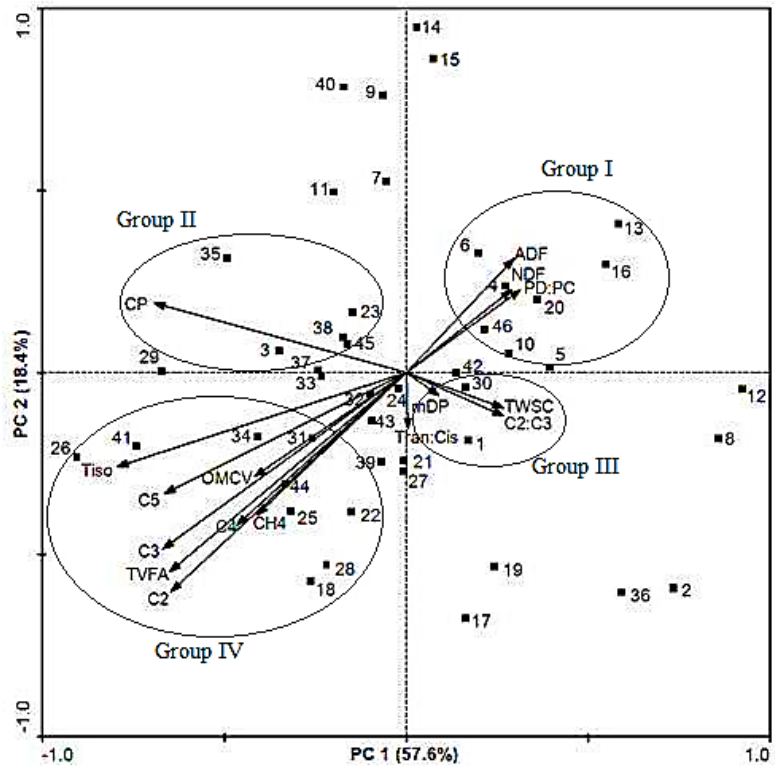

Figure 1. Principal component analysis (PCA) ordination diagram showing the position of each sainfoin accession in relation to the measured feed chemical composition, tannin composition, CH_4, GP and VFA. The numbers in the panel correspond to the sainfoin accessions as presented in Table 1. ADF, acid detergent fibre; CP, crude protein; mDP, mean degree of polymerisation; NDF, neutral detergent fibre; PD:PC, ratio of prodelphinidin:procyanindin in CTs; TVFA, total volatile fatty acids (C2 + C3 + C4 + C5 + Tiso); *trans:cis*, ratio of *trans*- and *cis*-flavanols in CTs; TWSC, total water-soluble carbohydrate; Tiso, total iso-acids (iso-butyrate + iso-valerate); C2, acetate; C3, propionate; C4, butyrate; C5, valerate; CH_4, methane and OMCV, gas production.

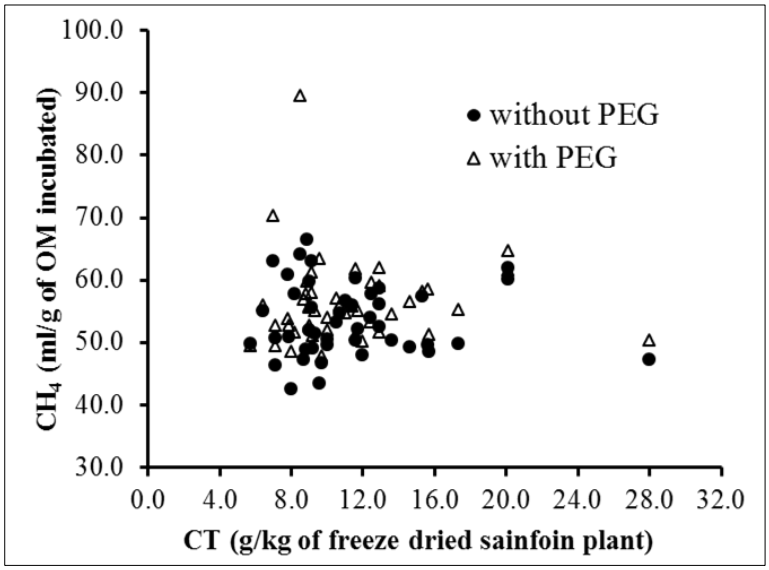

Figure 2. Relationship between condensed tannins (CT) content and in vitro methane production of sainfoin accessions incubated without (•) and with (Δ) polyethylene glycol (PEG) for 48 h.

DISCUSSION

Before the 1950's, sainfoin was grown much more widely in Europe because of its positive contributions to ecosystem services, i.e. enhancing soil fertility, attracting pollinating insects, and also to animal nutrition and health (Mueller-Harvey, 2009). Whilst several forage legumes have been the subject of intensive plant improvement programmes, sainfoin has not

yet been targeted in Europe although several opportunities have been identified (Doyle et al., 1984; Hayot Carbonero et al., 2011b). This study was part of the EU 'HealthyHay' project, which sought to characterise, identify promising candidates, and lay foundations for future sainfoin improvement programmes that will contribute towards developing sustainable ruminant feeding strategies. Therefore, accessions evaluated in the present study were not restricted to currently grown cultivars or varieties, but included also accessions, which were collected from different germplasm collections around the world (Table 1). The sainfoin collection that was established at the National Institute of Agricultural Botany (NIAB, Cambridge, UK), therefore, was representative of the currently available genetic diversity. This germplasm collection has been evaluated for agronomic performance (Hayot Carbonero, 2011b), tannin contents and composition (Stringano et al., 2012). The present study used the in vitro gas production technique for screening this germplasm collection for inhibitory properties of the different accessions on ruminal CH_4 production.

Nutritional analysis

The CP and fibre contents of most accessions were consistent with previous findings (Liu et al., 2008). On average, the NDF and CP contents in this previous study ranged from 435 to 488 g/kg of DM and 148 to 160 g/kg of DM, respectively, for the first harvest (April to September). The NDF in NIAB collection ranged from 338 to 585 g/kg of DM, whereas the CP contents ranged from 77 to 166 g/kg of DM, and meet the minimum required level (80 g/kg of DM) for optimal microbial activity in the rumen (Annison and Bryden, 1998) except accession 1041R2 which was marginal at 77 g CP/kg of DM.

Correlations between tannin content, composition and fermentation products

Generally, tannins have been associated with less CH_4 production because (i) they reduce fibre degradation by complexing with lignocellulose, which in turn prevents microbial fermentation, or (ii) by directly inhibiting cellulolytic microorganisms or a combination of both (McSweeney et al., 2001). To date, it is not known whether these effects are related to CT content or CT structures. The current study used PEG as a CT neutralising agent (Silanikove et al., 2001), which was expected to increase CH_4 production. Although PEG did increase CH_4 production, the increase was not related to CT content in all accessions (Table 4 and Figure 2). Several accessions with relatively high CT concentrations did not yield low CH_4 (−PEG treatment). In contrast, accessions 1012R2, 1077R2, 1104R2, and 1157R1 contained low CT concentrations (Table 2) produced high CH_4 in the presence of PEG

treatment (Table 4). This suggested that CT concentration was not obviously responsible. Other accessions provided further evidence. With accession 1156R2, the +PEG treatment produced the highest CH_4 increase at 6, 12, 24 and 48 h, but this accession had a relatively low CT content (8.5 g CT per kg of freeze dried sainfoin plant material). Accession 1256R1 with a relatively high CT content (28 g CT per kg of freeze-dried sainfoin plant material) did not produce much CH_4 in the presence of PEG (+PEG). Most surprisingly, for accession 1165R2 (20.1 g CT per kg of freeze-dried sainfoin plant material) no difference in CH_4 production was observed between –PEG and +PEG treatments. It is possible that CT structural features and the presence of other plant components, which may have interacted with CT, will have modified their activities. It is well known that CT interacts with proteins, cell walls, fibres and other compounds (Le Bourvellec et al., 2007; Bindon and Kennedy, 2011; Dobreva et al., 2011).

Gas production from a variety of feeds incubated in vitro is closely related to VFA production and is related to carbohydrate fermentation (Getachew et al., 2002). The VFA produced during enteric fermentation is a primary source of energy for ruminants. Besides, the type of VFA produced in the rumen determines CH_4 production. The formation of acetate in the rumen promotes CH_4 production, whereas propionate production and methanogenesis involves processes that compete for hydrogen (Moss et al., 2000). Therefore, quantification of VFA concentrations produced from fermentation of sainfoin accessions provides useful information for comparing their nutritional values. Total VFA and individual VFA concentrations were affected (decreased or increased) with +PEG treatment (Table 5). However, a significant increase ($P < 0.001$) upon +PEG treatment was observed only for propionic and valeric acids. In addition, no accession × PEG interaction effect on GP (Table 3) and CH_4 production was found (Table 4). In contrast, Azuhnwi et al. (2011) reported a significant increase in GP after adding PEG to sainfoin accessions. The difference could also be due to different plant maturity at harvest (Koupai-Abyazani et al., 1993; Theodoridou et al., 2011), which could affect the plant CT and nutritional compositions.

The accessions investigated in the present study differed greatly in CT structures in terms of tannin size (mDP values) and flavanol composition (Table 2). These sorts of differences can affect CT binding properties (Frazier et al., 2010) and thus digestion and nutritional value of forages (Mueller-Harvey et al., 2006). However, the mechanisms by which tannins reduce ruminal degradation of different dietary components have not yet been established. Sainfoin contains a heterogeneous mix of different tannin structures, which probably affects their

interactions with plant components. It may also yield complexes that vary in the extent of their ruminal protein protection. For instance, a higher PD:PC ratio reflects more hydrogen bonding sites, which may enhance the affinity of CT (Aerts et al., 1999) and thus slow down ruminal protein and fibre degradation. In agreement with this, the PCA analysis (Figure 1) showed a strong positive correlation between PD:PC and fibres but the correlation with protein was weak; this reflects the relative impacts of fibres and CP on the fermentation products in the presence of this sainfoin CT.

Principal component analysis for investigating multiple trait relationships

As differences in CT contents did not reveal clear positive or negative effects on GP (Table 3), CH_4 (Table 4) or VFA production (Table 5), the data were subjected to principal component analysis (PCA) in order to examine the relationships among multiple traits (CT composition, nutritional content, CH_4, GP and VFA). Principal component analysis showed correlations among forage composition and CT structural features with CH_4 production in some accessions (Figure 1). Even though individual constituents covered a wide range (Table 2), the relationships between CH_4 production and forage constituents were not conclusive. In some accessions, CH_4 production was negatively related to nutritional components such as NDF and ADF (Figure 1). The PCA loading plot showed that in some sainfoin accessions producing CH_4, production is negatively associated with variables that relate to forage quality (such as fibre) and to flavanol composition (e.g., PD:PC ratio). On the other hand, according to Taweel et al. (2005), fermentation of non-structural carbohydrates in forage diets generates more propionic acid. However, total-water soluble carbohydrate tended to show a weak relationship with both propionic acid and CH_4 production in some sainfoin accessions. This result agrees with the elevated water-soluble carbohydrate concentrations in perennial ryegrass cultivars, which were also not strongly related with in vitro total gas production, CH_4 production and organic matter digestibility (Lovett et al., 2004).

The present screening study suggested that the accessions collected across the world and evaluated under similar conditions exhibited substantial variation in terms of ruminal in vitro CH_4 production. However, sainfoin CT contents did not affect CH_4 production but other multiple factors appear also involved. Principal component analysis was able to cluster accessions into several groups according to their overall similarity, which might require further investigations. The grouping explained by the PD:PC ratio appears to contain an important source of variation that is negatively related to CH_4 production and, therefore, sainfoin accessions numbered 1012R2, 1017R2, 1026R2, 1043R2, 1103R2, 1123R2 and

1264R2 deserve further examinations. We conclude that during evaluation of different sainfoin accessions for their rumen in vitro CH_4 production focus should not only give to CT contents and macro nutrients, but should also take into account the CT structures.

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CHAPTER 7

Impact of variation in structure of condensed tannins from sainfoin (*Onobrychis viciifolia*) on in vitro ruminal methane production and fermentation characteristics

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ABSTRACT

Our study investigated the effects of condensed tannins (CT) on rumen in vitro methane (CH_4) production and fermentation characteristics by incubating lucerne in buffered rumen fluid without (control) or with CT extracts set at 40, 80 and 120 g CT per kg of substrate DM. Condensed tannins were extracted from four sainfoin accessions: Rees "A", CPI63763, Cotswold Common and CPI63767. Gas production (GP) was measured using a fully automated GP apparatus with CH_4 measured at distinct time points. Condensed tannins differed substantially in terms of polymer size and varied from 13 (Rees "A") to 73 (CPI63767) mean degree of polymerization, but had relatively similar characteristics in terms of CT content, procyanidin:prodelphinidin (PC:PD) and *cis:trans* ratios. Compared to control, addition of CT from CPI63767 and CPI63763 at 80 and 120 g CT per kg of substrate DM reduced CH_4 production by 43 and 65%, and by 23 and 57%, respectively, after 24 h incubation. Similarly, CT from Rees "A" and Cotswold Common reduced CH_4 production by 26 and 46%, and by 28 and 46%, respectively. Addition of increasing level of CT linearly reduced the maximum rate of gas and CH_4 production, and the estimated in vitro organic matter digestibility. There was a negative linear and quadratic ($P < 0.01$) relation between CT concentration and total volatile fatty acid (VFA) production. Inclusion of 80 and 120 g CT per kg of substrate DM reduced ($P < 0.001$) branched-chain VFA production and acetate:propionate ratio and was lowest for CPI63767. A decrease in proteolytic activity as indirectly shown by a change in VFA composition favouring a shift towards propionate and reduction in branched–chain VFA production varied with type of CT, and was highest for CPI63767. In conclusion, these results suggest that tannin polymer size is an important factor affecting in vitro CH_4 production, which may be linked to the CT interaction with dietary substrate or microbial cells.

Key words: sainfoin; condensed tannin; polymer size; methane; in vitro

INTRODUCTION

Plants produce a vast array of different tannin types and concentrations (Khanbabaee and Van Ree, 2001; Mueller-Harvey, 2006; Huemmer and Schreier, 2008). For a long time tannins have been considered as an anti-nutritional factor in animal nutrition (Mueller-Harvey, 2006). Whether tannins exert positive or negative effects appears to depend on the type and level of tannins in the plants (Barry and McNabb, 1999; Min et al., 2003), the amount ingested and the animal species involved (Frutos et al., 2004; Mueller-Harvey, 2006). It is of note, however, that only a few studies investigated their bioactivities and included a full tannin analysis. Most of these studies compared the procyanidin–rich *Lotus corniculatus* (birdsfoot trefoil) and the prodelphinidin–rich *Lotus pedunculatus* (big trefoil) tannin types (Molan et al., 2001; Min et al., 2003; Tavendale et al., 2005). However, condensed tannins (CT) vary considerably even within a single plant species (Koupai-Abyazani et al., 1993; Marais et al., 2000; Stringano et al., 2012). Studies are needed to test how this variation affects their biological activity in relation to ruminant nutrition, nitrogen and methane (CH_4) emission.

Tannins of various origins have been shown to inhibit ruminal CH_4 production either when fed to ruminants as tannin–containing forages (Woodward et al., 2001; Puchala et al., 2005) and as tannin extracts tested in vitro (Tavendale et al., 2005; Pellikaan et al., 2011b; Hassanat and Benchaar, 2013) or fed in vivo (Beauchemin et al., 2007; Animut et al., 2008; Bhatta et al., 2013). To date, this anti-methanogenic activity appears to be variable and could not be explained by simply grouping the tannins into hydrolysable or condensed tannin types (Bhatta et al., 2009; Pellikaan et al., 2011b), suggesting that specific chemical structural properties are responsible for their anti-methanogenic activity. However, to our knowledge, limited studies are available focusing on chemical structural composition of CT to elucidate which chemical property is most responsible in reducing ruminal CH_4 production.

The objective of this study was to investigate the structural variation of semi-purified condensed tannin extracts, which had been obtained from four sainfoin accessions on rumen in vitro CH_4 production and fermentation characteristics. We hypothesized that the mean degree of polymerization (i.e. polymer size) of the tannin molecule is the most important property determining its activity to inhibit in vitro CH_4 production.

MATERIAL AND METHODS

Plant samples for preparation of condensed tannin extracts

Four sainfoin accessions were selected from the EU 'HealthyHay' sainfoin germplasm collection based on their distinct differences in terms of CT structure (Stringano et al., 2012). The four accessions were accession number 1165 (Rees "A"), 1123 (CPI63763), 1262 (Cotswold Common) and 1127 (CPI63767). The plants were grown at the National Institute of Agricultural Botany (NIAB; Cambridge, UK). Growing conditions and source of seeds were reported previously (Hayot Carbonero et al., 2011). Sainfoin accessions were harvested when about 50% of stems showed open flowers on the lowest half of the flower stem. Plant material was packed in special bags (Nalgene low density polyethylene bags; 22.9 × 45.7 cm), stored at –20°C, freeze-dried and then ground to pass an 8-mm sieve using an impeller mill (Retsch GmbH, SM1, Haan, Germany), and subsequently ground to pass a 1-mm sieve (Retsch GmbH, ZM 100, Haan, Germany).

Extraction of condensed tannins

The CT extracts were prepared as described by Stringano et al. (2011). Briefly, 25 g of ground (1-mm) sainfoin sample was extracted once with acetone/water (200 ml; 7:3, v/v) containing ascorbic acid (1 g/l) for 40 min. Chlorophyll was removed from the acetone/water solution by extracting twice with dichloromethane (200 ml). Acetone was then removed on a rotary evaporator and the aqueous phase was concentrated in vacuum (< 40^{o}C), and subsequently freeze-dried to yield CT extracts. The extracts were stored at –20°C until use.

Analysis of condensed tannin extracts

Condensed tannin extracts were analysed for CT content and structural properties by thiolysis as described by Gea et al. (2011). Briefly, freeze-dried extract (4 mg) was weighed into a glass tube and 1 ml methanol was added, followed by 50 µl of acidified methanol (3.3 ml concentrated HCl in 100 ml methanol) and 100 µl benzylmercaptan in methanol (5:95, v/v). The reaction mixture was stirred at 40°C for 30 min. The reaction was stopped by cooling in an ice-water bath. Water (250 µl) and then dihydroquercetin in methanol (50 µl; 0.047 mg/ml) as the internal standard was added. Samples were then analysed by high performance liquid chromatography. This provided information on monomeric flavanol composition (Figure 1) and allowed calculation of the mean degree of polymerization (mDP), procyanidin:prodelphinidin (PC:PD) and *cis:trans* flavanol ratios (Gea et al., 2011; equations 1, 2 and 3).

$$\text{mDP} = \frac{\text{amount of extension and terminal flavanol units (mol)}}{\text{amount of terminal flavanol units (mol)}} \quad (1)$$

$$\text{PC:PD} = \frac{\text{percentage of C + EC units}}{\text{percentage of GC + EGC units}} \quad (2)$$

$$cis:trans = \frac{\text{percentage of EC + EGC units}}{\text{percentage of C + GC units}} \quad (3)$$

where C, catechin; EC, epicatechin; GC, gallocatechin; and EGC, epigallocatechin.

Substrate and condensed tannin preparations

Effects of CT on in vitro CH_4 production and fermentation kinetics were examined using the tannin-free lucerne (*Medicago sativa*) as a substrate. Lucerne was harvested at 50% flowering stage, freeze-dried and ground to pass a 1-mm sieve (Retsch GmbH, ZM 100, Haan, Germany). The chemical composition of lucerne was: OM = 800.0 g/kg of DM; CP = 188.0 g/kg of DM; NDF = 279.5 g/kg of DM and ADF = 211.2 g/kg of DM. Condensed tannins were prepared at 3 effective concentrations: 40, 80 and 120 g CT per kg of substrate DM. The extracts used in the present study differed in their CT contents and range from 5 to 11 g CT per 100 g extract (Table 1). Therefore, the amount of CT extract required to achieve these three effective CT concentrations was weighed separately into Eppendorf vials and dissolved in 2 ml of Millipore water (Milli-Q Academic, Amsterdam, The Netherlands) and added to the fermentation bottles at the onset of in vitro incubation. Condensed tannin extracts were dissolved in water to ensure its proper homogenization with the substrate.

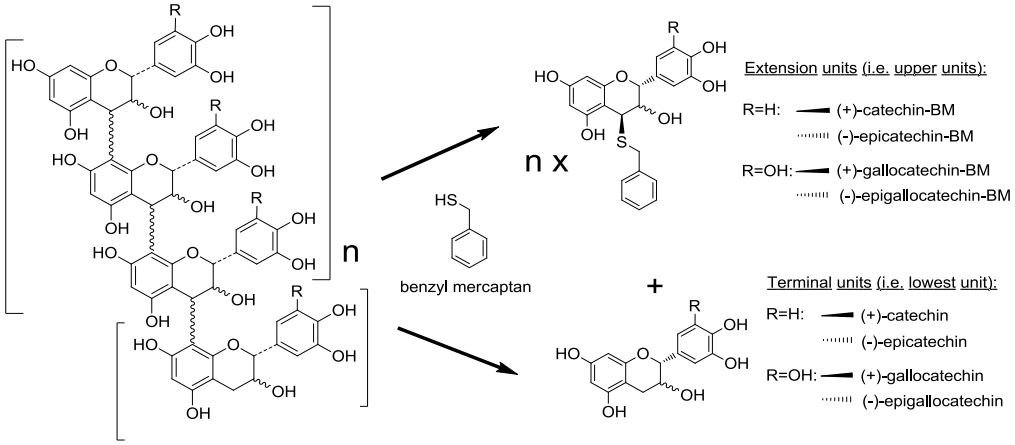

Figure 1. Structural features of condensed tannins.

Table 1. Condensed tannins (CT) content, mean degree of polymerization, PC:PD and *cis:trans* ratios in aqueous acetone extracts obtained from four sainfoin accessions[1].

CT type	CT content (g/100 g extract)	mDP[2]	PC:PD[3]	*cis:trans*[4]
Rees "A"	11 (2.2)	13 (2.3)	29:71 (1.4)	78:22 (4.4)
CPI63763	7 (1.9)	24 (6.7)	23:77 (2.6)	68:32 (3.9)
Cotswold Common	10 (1.5)	31 (1.6)	29:71 (0.7)	79:21 (1.7)
CPI63767	5 (0.9)	73 (3.0)	25:75 (2.0)	74:26 (2.0)

[1]Number in bracket indicates standard deviation.
[2]mDP, mean degree of polymerization (average number of flavanol monomers per tannin polymer).
[3]PC, procyanidin (i.e., CT that contain catechin and epicatechin units); PD, prodelphinidin (i.e., CT that contain gallocatechin and epigallocatechin units).
[4]*cis:trans,* the orientation of functional groups within a molecule.

Rumen in vitro gas and methane production measurements

Cumulative gas production (GP) was measured using a fully automated time related GP apparatus (Cone et al., 1996) and CH_4 production at distinct time points as described by Pellikaan et al. (2011a). Approximately 250 mg of substrate was weighed into 250 ml fermentation bottles (Schott, Germany). Bottles were then randomly distributed within each GP unit, such that bottles with each substrate–CT extract treatment combination including the blanks were incubated in each GP unit. Rumen fluid was obtained from three ruminally fistulated lactating Holstein–Friesian dairy cows. Donor cows were fed a grass and maize silage mixture in the morning and afternoon and 7−8 kg/d of concentrate according to their milk production. The handling of the animals was approved by the institutional animal care and use committee of Wageningen University (Wageningen, The Netherlands) and in accordance with the Dutch legislation on the use of experimental animals.

Rumen fluid was collected prior to the morning feeding by suction method using a solid perforated plastic tube (85 cm long and 2.5 cm in diameter). Rumen fluid once collected was transferred into pre–warmed and carbon dioxide (CO_2) flushed thermos flasks, transported quickly to the laboratory, pooled and filtered through two layers of cheesecloth into a flask flushed with CO_2. Filtered rumen fluid was mixed with the buffered mineral solution at 1:2 ratios (v/v) as described by Cone et al. (1996) with constant stirring and continuous flushing with CO_2, while maintained in a water bath set to 39^{o}C. Then, 30 ml buffered rumen fluid mixture was subsequently dispensed in the fermentation bottle prewarmed to 39^{o}C. Finally, CT solution was immediately added into the fermentation bottle and incubated in a water bath maintained at 39^{o}C and shaking at 40−50 movements per minute. Control bottles containing

substrate and buffered rumen fluid (i.e. without CT) were injected with 2 ml of Millipore water.

The study was designed as a randomized complete block design with incubation run considered as a block. Each treatment and control were incubated in duplicate within a run and replicated in two runs on different days. Two bottles in each run were included as a blank (containing only buffered rumen fluid) and GP for each bottle was corrected for the blank values. The amount of gas, CH_4 and VFA produced were adjusted to the total amount of organic matter (OM) incubated and expressed per gram of incubated OM (substrate OM plus extra OM supplied with CT extracts).

Methane concentration in the headspace of the fermentation bottle was measured by gas chromatography (GC; GC8000Top, CE Instruments, Milan, Italy). To allow gas sampling from the headspace, the fermentation bottles were fitted with a side port sealed with a screw cap that is fitted with an air-tight septum (GRACE, XLB-11 Septa 7/16, Breda, The Netherlands) as illustrated by Pellikaan et al. (2011a). At distinct time points of incubation (0, 2, 4, 6, 8, 10, 12, 24, 26, 28, 30, 32, 48, 50, 52, 54 and 72 h), 10 µl aliquots of the bottles headspace gas were sampled through this opening using a gas tight syringe (Gastight® # 1701 Hamilton 1701N, 10 µl Syringe, Point Style 5, Bonaduz, Switzerland) and analysed for CH_4 concentration using GC. The GC was fitted with a flame ionization detector and stainless steel column (6 m long, 0.53 mm i.d., 25 µm film thicknesses) packed with PoraPack Q 50−80 mesh (GRACE, Breda, The Netherlands). The temperatures of the injector, column, and detector were maintained at 150, 60 and 150°C, respectively. The carrier gas was nitrogen, and the pressure for nitrogen, hydrogen and air was set at 100, 50 and 100 kPa, respectively. The CH_4 concentration was calculated by external calibration, using a certified gas mixture containing known composition of CH_4 (Linde Gas Benelux, Schiedam, The Netherlands). Peak areas were determined by automatic integration system software (Chrom−Card data system Version 2.4, 2006, Rodano Milan, Italy) for GC.

Cumulative CH_4 production was calculated following the procedure described by Pellikaan et al. (2011b; equation 4) by taking the sum of the increased amount of CH_4 in the bottle headspace between two successive valve openings and the amount of CH_4 vented from the bottle.

$$\mathrm{M} = \sum_{i=1}^{n}\{\mathrm{V}_{HS}(\mathrm{C}_{i+1} - \mathrm{C}_i) + \mathrm{G}_{i+1}\mathrm{C}_{i+1}\} \quad (4)$$

where M, cummulative CH_4 production (ml/g of incubated OM); V_{HS}, the bottle headspace volume (ml); C_i and C_{i+1}, CH_4 concentration in the bottle headspace gas at i and i+1 valve openings, respectively; G_{i+1}, the amount of gas (ml) vented at i+1 valve opening; and n, total number of valve openings.

Curve fitting and calculations

Cumulative gas and CH_4 production curves were fitted iteratively with a triphasic and monophasic Michaelis–Menten equation (Groot et al., 1996; equation 5), respectively, using the non-linear least squares regression procedure in SAS (SAS, 2010).

$$\mathrm{OMCV} = \sum_{i=1\ or\ 3}^{n} \frac{\mathrm{A}_i}{1 + (\mathrm{B}_i/\mathrm{t})^{\mathrm{C}_i}} \quad (5)$$

where OMCV, gas or CH_4 production (ml/g of incubated OM); A, the asymptotic gas production (ml/g of incubated OM); B, time at which half of the asymptotic gas or CH_4 production has been reached ($t_{1/2}$, h); C, the sharpness of the switching characteristics of the profile; and t, the time (h).

The maximum rate of gas or CH_4 production (R_{max}, ml/h) was calculated as described by Bauer et al. (2001; equation 6).

$$\mathrm{Rmax} = \frac{\mathrm{A} \times \mathrm{B}^{\mathrm{C}} \times \mathrm{C} \times \mathrm{TRmax}^{(-\mathrm{C}-1)}}{(1 + \mathrm{B}^{\mathrm{C}} \times \mathrm{TRmax}^{-\mathrm{C}})^2} \quad (6)$$

where A, asymptote gas or CH_4 production (ml/g of incubated OM); B, time of incubation at which half of the asymptote gas or CH_4 has been formed ($t_{1/2}$, h); C, the sharpness of the switching characteristic of the profile.

In vitro organic matter digestibility

The in vitro organic matter digestibility (IVOMD) was estimated according to the equation given by Menke and Steingass (1988; equation 7) based on 24-h gas production and nutrient composition of the substrate.

$$\mathrm{IVOMD}\ (\%) = 14.88 + 0.8893 \times \mathrm{GP} + 0.0448 \times \mathrm{CP} + 0.0651 \times \mathrm{A} \quad (7)$$

where GP, is 24-h net gas production (ml/200 mg of DM); CP, is crude protein (%) and A, is ash (%) contents of the substrate.

Analytical procedures

Substrate sample was freeze-dried, ground using a Wiley mill through a 1-mm sieve and analysed for DM (ISO 6496, 1999), ash (ISO 5984, 2002) and N (ISO 5983, 2005). Crude protein content was calculated as: CP = 6.25 × N. Neutral detergent fibre and ADF were analysed using an ANKOM^{2000} Fibre Analyzer (ANKOM Technology Corporation, Macedon, NY, USA).

The VFA sample (750 µl) from each bottle after 72 h incubation was acidified with equal volume of 0.85% orthophosphoric acid containing 19.68 mM isocaproic acid as internal standard, and stored at –20^{o}C pending for further analysis. The VFA concentration was analysed by GC as described by Taweel et al. (2005). The VFA concentration in the medium was corrected for the VFA concentration of blank (i.e. rumen fluid plus buffer) and expressed as mM per gram of incubated OM.

Statistical analysis

All duplicate bottles per treatment within run were averaged prior to statistical analysis. Fermentation bottle was considered as an experimental unit. Data were subjected to analysis of variance based on a complete randomized design within a 4 × 4 factorial arrangement of treatments using the GLM procedure in SAS (SAS Institute Inc., 2010). For each CT type, the effects of CT concentration on gas, CH_4, VFA and kinetic parameters were analysed for orthogonal polynomial contrasts. The model included treatment (CT level) as a fixed effect and block (run) as a random effect. Least square means for control and treatments are reported. Treatment effects were declared significant at $P \leq 0.05$ and tendency at $0.05 < P \leq 0.10$.

RESULTS

Chemical composition of lucerne and chemical characteristics of condensed tannins

The analysed organic matter (OM) content of lucerne was somewhat lower than the expected. Since GP, CH_4 production and kinetic parameters were expressed per unit of OM for all treatments; the lower OM content of the lucerne does not affect differences between treatments. There was a large variation in CT average polymer sizes (mDP) among the four CT types but much less variation in content, PC:PD and *cis:trans* ratios (Table 1). The mDP values varied from 13 to 73 (Rees "A" vs. CPI63767) and the CT content from 5 to 11 g CT per 100 g extract (CPI63767 vs. Rees "A"). The PC:PD ratio ranged from 23:77 to 29:71

(CPI63763 vs. Rees "A" and Cotswold Common) and *cis:trans* ratio from 68:32 to 79:21 (CPI63763 vs. Cotswold Common).

Effect of condensed tannins on total gas and methane production

Cumulative gas (ml/g of incubated OM) was reduced by the type and level of CT compared with the control (Table 2). A linear ($P < 0.001$) reduction was observed with increasing CT level for all CT type after 72 h. On average less gas was produced by inclusion of CT from CPI63767 and followed by Cotswold Common, Rees "A" and CPI63763 CT at 12, 24 and 72 h. Condensed tannins from CPI63767 when added at ≥ 80 g CT/kg of substrate DM consistently gave the lowest GP at 12, 24 and 72 h compared with the control and the other CT types. All types of CT when added at 80 and 120 g CT/kg of substrate DM have linearly decreased GP.

The effect of CT on CH_4 production is presented in Table 3. Condensed tannins from CPI63767 were the most effective in reducing CH_4 production followed by CT from CPI63763, Rees "A", and Cotswold Common. Addition of CT at 40 g CT/kg of substrate DM, except CPI63767, did not affect CH_4 production. Inclusion of CT from CPI63767 at 80 and 120 g CT/kg of substrate DM reduced ($P < 0.001$) CH_4 by 43 and 65% compared with the control after 24 h incubation. Similarly, CT from CPI63763 reduced CH_4 ($P < 0.001$) by 23 and 57% after 24 h, while Rees "A" and Cotswold Common reduced CH_4 by about 26 and 46%, and 28 and 46%, respectively. Inclusion of CT at 120 g CT/kg of substrate DM reduced CH_4 production by 28% (Rees "A" and Cotswold Common) and by 63% (CPI63767) compared with the control after 72 h of incubation. Methane production expressed per unit IVOMD was 33.6 ml/g of OM degraded for control, and 28.6, 20.4 and 12.5 ml/g of OM degraded for 40, 80 and 120 g CT/kg of substrate DM, resulting in a 15, 39 and 63% reduction for the respective CT levels (Table 3).

More than 50% of total CH_4 was produced in the first 12 h of incubation for the control treatment, which was considerably more than when substrate was incubated with CT. The proportions of CH_4 in total GP (v/v) showed a linear reduction ($P < 0.001$) for CPI63767 (21.9 – 12.1%) and CPI63763 (21.9 – 14.7%), and a linear and quadratic effect for Cotswold Common and Rees "A" after 72 h incubation (Figure 2). A higher proportion of CH_4 for Rees "A" (22.8%) and Cotswold Common (24.3%) were measured when CT was added at 40 g CT/kg of substrate DM compared with control (21.9%) after 72 h of incubation (Table 3). The same trend was observed after 24 h of incubation. Depending on the type of CT, CH_4 produced as a proportion of total gas during 24 h incubation varied from 18.6% (control) to

Table 2. Effect of type and concentration of sainfoin condensed tannins (CT) on in vitro gas production (ml/g of incubated OM) and in vitro organic matter digestibility of lucerne.

CT type	CT concentration (g/kg of substrate DM)	Time of post incubation (h)				IVOMD (%)[1]
		6	12	24	72	
Rees "A"	0	216.9	257.3	290.9	309.8	62.1
	40	188.0	226.6	257.1	271.1	54.7
	80	171.4	227.9	258.6	279.6	54.9
	120	149.5	205.4	228.0	243.5	50.4
	SEM[2]	3.92	3.91	5.10	5.48	0.76
	Linear	<0.001	<0.001	<0.001	<0.001	<0.001
	Quadratic	0.400	0.321	0.763	0.823	0.055
CPI63763	0	216.9	257.3	290.9	309.8	62.1
	40	199.0	251.2	290.0	308.5	59.6
	80	185.9	237.0	268.1	290.5	56.4
	120	159.4	205.4	227.1	239.6	50.3
	SEM	4.24	4.34	4.52	5.99	0.67
	Linear	<0.001	<0.001	<0.001	<0.001	<0.001
	Quadratic	0.335	0.085	0.165	0.212	0.091
Cotswold Common	0	216.9	257.3	290.9	309.8	62.1
	40	186.9	237.1	268.6	284.1	56.2
	80	162.4	217.8	242.0	258.0	52.3
	120	145.1	202.3	227.6	242.0	50.2
	SEM	4.58	3.12	3.25	4.51	0.49
	Linear	<0.001	<0.001	<0.001	<0.001	<0.001
	Quadratic	0.201	0.475	0.257	0.315	0.102
CPI63767	0	216.9	257.3	290.9	309.8	62.1
	40	187.6	239.9	273.9	292.3	57.3
	80	160.7	206.1	231.9	251.6	51.1
	120	144.6	181.8	199.6	206.5	46.3
	SEM	3.50	3.69	4.20	5.76	0.62
	Linear	<0.001	<0.001	<0.001	<0.001	<0.001
	Quadratic	0.387	0.374	0.099	0.638	0.959

[1]IVOMD, In vitro OM digestibility was determined based on 24-h gas production and chemical composition of the substrate (Menke and Steingass, 1988). [2]SEM, standard error of the means.

13.2% (CPI63767), 15.5% (CPI63763), 15.5% (Rees "A") and 16.0% (Cotswold Common) when CT was added at 80 g CT/kg of substrate DM.

Table 4 presents a set of substrate fermentation kinetics parameters. Asymptotic GP for the first phase (A1; the first 3 h of incubation, which corresponds to fermentation of the soluble and rapidly fermentable fraction; Groot et al., 1996) decreased linearly ($P < 0.010$) with increasing level of CT for all CT type. The asymptotic GP for the second phase (A2; 3−20 h of incubation, which corresponds to fermentation of the non-soluble fraction) only showed a linear ($P < 0.010$) decrease for Cotswold Common and CPI63767. The asymptotic GP for the third phase (A3; 20−72 h of incubation, corresponds to microbial turn over) was quadratically ($P < 0.010$) affected by CT from CPI63767. The rate of GP (R_{max1}) decreased linearly ($P < 0.050$) with increasing level of CT, but was unaffected for CPI63763. The half time of asymptotic GP in the first phase (B1) was longer for CT from Cotswold Common compared to control. In general, all CT types gave a longer B1 when added at 80 and 120 g CT/kg of substrate DM compared with the control. Similarly, increasing CT level from CPI63767 caused a higher reduction ($P < 0.010$) of both R_{max1} and asymptotic CH_4 production. For all CT types, the B1 of the asymptotic CH_4 production was affected in a quadratic manner ($P < 0.001$). The asymptotic CH_4 production was also affected quadratically, but only for CPI63767. On average, half of the asymptotic CH_4 production was reached after 18.6 h (CPI63767), 18.8 h (CPI63763), 37.3 h (Rees "A") and 25.6 h for Cotswold Common as compared with 8.9 h for the control.

Effects of condensed tannins on volatile fatty acids

Condensed tannins had linear and quadratic effects on total and individual VFA production (Table 5). There was a linear and quadratic relation between CT level and VFA production for CPI63767, and linear effect for Cotswold Common. The proportion of propionate linearly increased at the expense of acetate, and butyrate decreased for all CT types and levels of inclusion compared to the control. The highest increase in propionate production was observed for CT from CPI63767 added at 120 g CT/kg of substrate DM. A similar increase in propionate was observed when CT was added at 80 g CT/kg of substrate DM. The ratio of acetate: propionate was the lowest for the same CT when added at 80 and 120 g CT/kg of substrate DM. The reduction in ace:pro ratio was 20 and 40% when CT was added at 80 and 120 g CT/kg of substrate DM, respectively, compared with only 3% reduction when CT was added at 40 g CT/kg of substrate DM. On average the decline (% rel-

Table 3. Effect of type and concentration of sainfoin condensed tannins (CT) on in vitro methane production (ml/g of incubated OM); proportion of methane in total gas and methane produced per unit estimated OM degraded (ml/g of degraded OM).

CT type	CT concentration (g/kg of substrate DM)	Time of post incubation (h)				CH_4 per total gas[1]	CH_4 per IVOMD
		6	12	24	72		
Rees "A"	0	26.7	40.7	54.0	68.0	18.6	33.6
	40	22.1	35.6	50.9	61.8	20.0	28.1
	80	18.0	28.1	40.2	59.3	15.5	22.0
	120	12.5	19.3	28.9	49.0	12.7	14.6
	SEM[2]	1.25	1.25	0.66	3.99	0.46	0.39
	Linear	<0.001	<0.001	<0.001	0.012	<0.001	<0.001
	Quadratic	0.737	0.196	0.321	0.622	0.002	0.055
CPI63763	0	26.7	40.7	54.0	68.0	18.6	33.6
	40	22.6	36.4	51.2	66.8	17.6	30.5
	80	17.4	29.4	41.5	56.5	15.5	23.5
	120	10.6	16.6	23.3	35.2	10.3	11.7
	SEM	1.65	2.04	2.42	4.14	0.66	1.40
	Linear	<0.001	<0.001	<0.001	<0.001	<0.001	0.001
	Quadratic	0.437	0.070	0.073	0.442	0.199	0.104
Cotswold Common	0	26.7	40.7	54.0	68.0	18.6	33.6
	40	22.6	37.6	52.1	69.0	19.4	29.3
	80	12.9	30.9	38.8	53.3	16.1	20.3
	120	11.5	19.1	29.4	49.1	12.9	14.8
	SEM	1.59	1.67	0.76	3.35	0.41	0.32
	Linear	<0.001	<0.001	<0.001	0.002	<0.001	<0.001
	Quadratic	0.432	0.340	0.468	0.088	0.001	0.125
CPI63767	0	26.7	40.7	54.0	68.0	18.6	33.6
	40	20.1	32.9	46.3	61.0	16.9	26.5
	80	13.8	21.5	30.7	46.6	13.3	15.7
	120	9.1	13.9	18.9	24.9	9.5	8.7
	SEM	1.30	1.70	1.71	3.49	0.56	0.81
	Linear	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
	Quadratic	0.493	0.962	0.261	0.074	0.086	0.924

[1]Proportion of CH_4 in total gas (%) estimated based on 24-h incubation.

[2]SEM, standard error of the means.

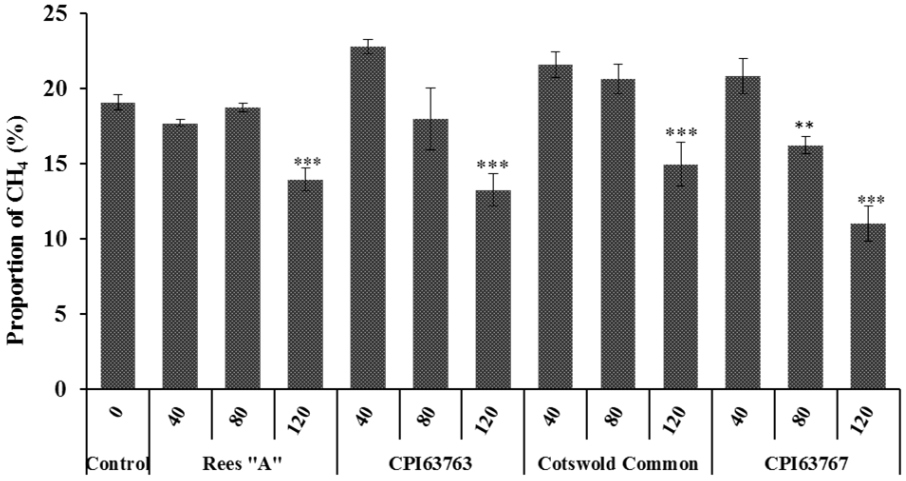

Figure 2. The effect of type and concentration of CT on the proportion of CH_4 production (expressed as percent of total gas) compared with the control after 72-h incubation (**P < 0.01; ***P < 0.001). The error bar shows the standard error of the means.

ative to control) in acetate:propionate ratio was 29% (CPI63767), 22% (CPI63763), 17% (Rees "A") and 16% for Cotswold Common.

DISCUSSION

Chemical property of condensed tannins

Condensed tannins (CT) from sainfoin (*Onobrychis viciifolia*) are rich in prodelphinidins and have a considerable spread of molecular weights, PC:PD ratio and tannin contents (Marais et al., 2000; Regos et al., 2009; Stringano et al., 2012). The "HealthyHay" sainfoin germplasm collection provided a unique opportunity to obtain contrasting CT in terms of molecular weights but otherwise relatively similar characteristics in CT content, PC:PD and *cis:trans* ratios (Table 1). Non-tannin components in acetone/water extracts consist of soluble carbohydrates (mainly sucrose), monomeric flavonoids and their glycosides and phenolic acids (Marais et al., 2000; Regos et al., 2009). These semi-purified CT extracts were used in the current study in order to assess the effects of different sainfoin CT types on rumen in vitro CH_4 production and fermentation characteristics. Previous approaches have tested commercially available tannins, which were either water or alcohol extracts, and found that the potential to reduce CH_4 production, or affect rumen fermentation and protein degradation varied with type and level of tannins (Getachew et al., 2008; Hassanat and Benchaar, 2013). Similarly, other studies, in which acetone/water extracts were added to in vitro systems, reported the effects of tannins on CH_4 production and their role in animal feed

Table 4. Effect of type and concentration of sainfoin condensed tannins (CT) on in vitro methane production and fermentation kinetics parameters of lucerne.

CT type	CT concentration (g/kg of substrate DM)	Methane production kinetics parameters			Gas production kinetic parameters[1]								
		A1	B1	R_{max1}	A1	A2	A3	B1	B2	B3	R_{max1}	R_{max2}	R_{max3}
Rees "A"	0	67.4	8.9	4.9	157.3	123.9	25.5	2.0	5.1	7.4	100.8	19.7	2.4
	40	71.1	13.9	4.0	129.5	106.2	23.6	1.6	4.1	21.9	86.2	16.4	2.0
	80	84.2	27.2	2.9	122.4	141.4	31.0	3.2	4.9	10.3	66.1	15.2	1.5
	120	87.1	70.6	1.3	98.1	98.4	18.8	1.0	5.4	11.3	50.5	13.1	1.3
	SEM[2]	6.61	9.77	1.00	7.76	12.41	3.18	1.40	2.05	6.39	14.36	1.33	0.27
	Linear	0.095	0.011	0.010	0.006	0.366	0.295	0.236	0.623	0.918	0.021	0.003	0.007
	Quadratic	0.890	0.113	0.536	0.833	0.060	0.042	0.025	0.925	0.032	0.973	0.633	0.812
CPI63763	0	67.4	8.9	4.9	157.3	123.9	25.5	2.0	5.1	7.4	100.8	19.7	2.4
	40	85.3	13.7	3.6	142.4	139.3	31.0	1.0	4.8	19.5	68.9	16.3	2.2
	80	67.6	14.9	3.0	133.5	136.7	30.9	1.3	4.5	16.0	73.2	14.2	1.3
	120	52.8	27.8	1.7	105.4	123.7	18.1	2.3	4.0	10.3	53.3	18.9	3.5
	SEM	5.09	3.59	0.96	6.22	2.37	3.06	0.69	0.72	2.19	15.76	1.76	0.64
	Linear	0.045	0.028	0.032	0.004	0.103	0.120	0.172	0.208	0.439	0.067	0.554	0.376
	Quadratic	0.063	0.265	0.236	0.240	0.004	0.052	0.088	0.045	0.013	0.668	0.040	0.088
Cotswold Common	0	67.4	8.9	4.9	157.3	123.9	25.5	2.0	5.1	7.4	100.8	19.7	2.4
	40	78.7	13.2	3.4	136.9	135.5	25.4	2.9	3.3	10.3	82.8	16.4	1.5
	80	53.7	17.0	2.5	96.1	110.2	21.3	3.3	3.7	11.0	63.4	13.9	1.8
	120	74.6	46.6	1.5	90.2	98.5	19.7	3.1	4.3	9.3	47.1	12.7	1.1

Table 4. (continued)

CT type	CT concentration (g/kg of substrate DM)	Methane production kinetics parameters			Gas production kinetic parameters[1]								
		A1	B1	R_{max1}	A1	A2	A3	B1	B2	B3	R_{max1}	R_{max2}	R_{max3}
	SEM	4.34	2.07	1.20	7.20	4.70	6.09	1.91	2.49	6.46	17.19	1.44	0.28
	Linear	0.592	<0.001	0.001	0.002	0.005	0.391	0.715	0.921	0.502	0.036	0.003	0.010
	Quadratic	0.023	0.003	0.365	0.367	0.012	0.997	0.016	0.794	0.248	0.916	0.499	0.765
CPI63767	0	67.4	8.9	4.9	157.3	123.9	25.5	2.0	5.1	7.4	100.8	19.7	2.4
	40	75.3	13.7	3.7	140.1	140.8	30.1	2.8	4.8	15.9	78.1	14.9	2.0
	80	72.0	29.7	2.9	113.2	120.0	26.7	2.2	6.3	6.5	75.9	27.4	6.2
	120	30.7	13.1	1.2	93.5	108.5	11.1	2.3	3.8	9.9	25.6	13.2	0.9
	SEM	4.85	1.56	1.06	5.12	1.35	1.90	1.36	0.73	3.76	17.65	1.34	1.16
	Linear	0.005	0.023	<0.001	0.001	<0.001	0.004	0.353	0.283	0.923	0.015	0.250	0.859
	Quadratic	0.009	0.003	0.460	0.962	0.002	0.008	0.132	0.097	0.022	0.490	0.004	<0.001

[1]A, asymptotic gas or CH_4 production (A1, A2, A3 indicates different phases; ml/g of incubated OM); B, half time of asymptotic gas or CH_4 production (B1, B2, B3 indicates different phases; h); R_{max} = rate of maximum gas or CH_4 production (R_{max1}, R_{max2}, R_{max3} indicates different phases; ml/h).

[2]SEM, standard error of the means.

Table 5. Effect of type and concentration of sainfoin condensed tannins (CT) on total VFA production and molar proportions of individual VFA.

CT type	CT (g/kg of substrate DM)	Total VFA (mM/g OM)	Individual VFA (mol/100 mol)[1]					Ace:Pro
			Ace	Pro	But	Val	BCVFA	
Rees "A"	0	5.29	65.53	23.33	7.00	1.30	2.83	2.83
	40	5.37	67.10	25.35	4.75	1.23	1.63	2.65
	80	4.94	67.55	27.43	3.45	1.13	0.50	2.50
	120	5.13	63.38	33.33	2.30	1.15	-0.15	1.93
	SEM[2]	0.112	0.719	0.939	0.155	0.037	0.098	0.119
	Linear	0.100	0.087	<0.001	<0.001	0.006	<0.001	<0.001
	Quadratic	0.609	0.002	0.161	0.154	0.199	0.106	0.120
CPI63763	0	5.29	65.53	23.33	7.00	1.30	2.83	2.83
	40	5.34	67.63	24.68	4.30	1.78	1.65	2.75
	80	5.72	65.90	29.23	2.43	2.03	0.45	2.25
	120	5.24	60.00	37.48	1.28	2.05	-0.80	1.60
	SEM	0.134	0.644	0.690	0.076	0.042	0.072	0.080
	Linear	0.689	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
	Quadratic	0.069	<0.001	0.201	0.312	<0.001	0.611	0.064
Cotswold Common	0	5.29	65.53	23.33	7.00	1.30	2.83	2.83
	40	5.17	68.53	23.73	4.90	1.15	1.70	2.90
	80	4.98	66.13	29.40	3.18	1.00	0.30	2.28
	120	4.96	63.83	33.08	2.33	0.98	-0.20	1.95
	SEM	0.093	0.674	0.833	0.124	0.028	0.075	0.118
	Linear	0.014	0.029	<0.001	<0.001	<0.001	<0.001	<0.001
	Quadratic	0.600	0.002	0.373	0.181	0.045	0.213	0.035
CPI63767	0	5.29	65.53	23.33	7.00	1.30	2.83	2.83
	40	5.56	67.80	25.73	3.70	1.45	1.35	2.65
	80	5.29	63.98	32.08	2.18	1.50	0.25	2.00
	120	4.52	56.68	42.15	1.00	1.43	-1.28	1.35
	SEM	0.117	0.528	0.685	0.123	0.028	0.081	0.097
	Linear	<0.001	<0.001	<0.001	<0.001	0.005	<0.001	<0.001
	Quadratic	0.001	<0.001	0.321	0.172	0.002	0.763	0.180

[1]VFA, volatile fatty acid; Ace, acetate; Pro, propionate; But, butyrate; Val, valerate; BCVFA, branched-chain VFA (iso-butyrate + iso-valerate).
[2]SEM, standard error of the means.

vary depending on the type and concentrations of tannins (Sivakumaran et al., 2006; Beauchemin et al., 2007; Pellikaan et al., 2011b).

The CT used in the present study consisted mainly of prodelphinidins and a wide polymer size (mDP values) ranging from 13 to 73 (Table 1). However, the literature contains surprisingly contradictory information on the effect of tannin polymer size on anti-methanogenic properties. Tavendale et al. (2005) found that CT polymers with a mDP value of 12.5 (DP range from 4 – 13) completely inhibited methane production, but CT oligomers with mDP values of 4.5 to 6.6 (DP range from 2 – 7) had no inhibitory effect. In contrast, Field et al. (1989) found that autoxidized oligomers in particular had anti-methanogenic effects, while their autoxidized high molecular weight polymers showed no inhibiting effects. These seeming contradictions could arise from the fact that the autoxidized polymers from Field et al. (1989) are unlike the naturally occurring plant polymers studied by Tavendale et al. (2005). Another explanation could be that condensed tannins are highly specific in their anti-methanogenic properties just as reported previously for hydrolysable tannins (Pellikaan et al., 2011b) and for condensed tannins (Hassanat and Benchaar, 2013). It is also important to note that mDP values describe the 'average CT' in a mixture of different tannin molecules and thus the CT distribution profiles will include tannins of different molecular weights (Stringano et al., 2011).

Effect of condensed tannins on in vitro methane and VFA production

The inclusion of CT from Rees "A" having the lowest polymer size (mDP = 13) compared with CT from CPI63767 that has the highest polymer size (mDP = 73) at the same concentration affected CH_4 production differently (Table 3). We note that both CT types have relatively similar PC:PD ratio (29:71 and 25:75, respectively). The inclusion of CT from these two sources at 80 g CT/kg of substrate DM produced 59.3 and 46.6 ml CH_4/g of incubated OM for CT from Rees "A" vs. CPI63767 after 72 h of incubation (Table 3). The same trend was observed when CT was added at 120 g CT/kg of substrate DM and incubated for 6, 12, and 24 h. Similarly, inclusion of CT from CPI63767 (mDP = 73; PC:PD = 25:75) compared to CT from CPI63763 (mDP = 24; PC:PD = 23:73) at the same concentration, for instance, 80 g CT/kg of substrate DM, reduced CH_4 production to different extent. This suggests that the PC:PD ratio at least for the type of CT tested in the present study is not the main responsible factor explaining the differences observed in their potential of reducing in vitro CH_4 production. In contrast, Molan et al. (2001) observed that CT extracts from prodelphinidin-rich big trefoil (*Lotus pedunculatus*) was more active in inhibiting the growth of proteolytic

rumen bacteria than procyanidin-rich birdsfoot trefoil (*Lotus corniculatus*). The inconsistencies between studies could be due to difference in source of CT extracts (i.e. plant of origin) that might affect their activity to bind carbohydrates and/or proteins (McAllister et al., 2005).

The ratio of CH_4 to total gas is an important indicator of the potential amount of CH_4 produced per unit OM degraded. The proportion of CH_4 in the total gas on average declined from 18.6% to 11.4%; a reduction of 39% (control vs. 120 g CT/kg of substrate DM; Table 3). This is in agreement with Hassanat and Benchaar (2013), who reported up to 40% reduction of CH_4 production compared with control when substrate was incubated with condensed tannins at ≥ 100 g/kg with minimum detrimental effects on efficiency of ruminal fermentation. Similarly, Waghorn et al. (2002) found that CH_4 production was reduced by 31% when sheep were fed on *Lotus pedunculatus* (DMI = 935 g/day; CT content = 5.3 g/100 g DM) compared with *Medicago sativa*, a tannin-free legume. In the present study, addition of CT at 40 g CT/kg substrate DM from Rees "A" and Cotswold Common did not inhibit CH_4 production (Table 3). However, at increasing CT levels CH_4 production was inhibited. This is in consistence with a meta-analysis conducted by Jayanegara et al. (2011) who showed a significant negative correlation between CT concentration and in vitro CH_4 production. Equally, McMahon et al. (1999) demonstrated that there is a linear suppression of in vitro CH_4 production with an increasing proportion of sainfoin forage in lucerne.

Regardless of the CT type, substrate incubated with ≥ 80 g CT/kg substrate DM was less degraded than the control as reflected by a lower in vitro OM digestibility (IVOMD; Table 2). In agreement Kaplan (2011), who compared the in vitro ruminal degradability of four accessions of sainfoin hay (containing extractable CT varying from 49 to 100 g/kg DM), found that estimated IVOMD of sainfoin hay is negatively correlated with its CT content. In contrast, Theodoridou et al. (2011) found that sainfoin CT extracted from whole-plant, leaves and stems (CT content 13.6, 9.8 and 9.0 g/kg of DM for whole-plant, leaves and stems, respectively) inhibited in vitro CH_4 production without altering its measured OM digestibility. The variations among studies could be due to the following: (i) in the study of Theodoridou et al. (2011), the IVOMD was measured, whereas in current study and that of Kaplan (2011), it was estimated from gas production and chemical composition of the substrate or (ii) lower CT content in the study of Theodoridou et al. (2011).

Addition of 120 g CT/kg of substrate DM showed an average reduction of 24% in 24 h GP and a 63% reduction in CH_4 expressed per unit of IVOMD. This relationship between the

reduction in CH_4 production and CT concentration suggests that the effects of CT may be attributed for an important part to a negative effect on ruminal fibre degradation such as increased formation of tannin–cellulose complexes that are resistant to enzymatic digestion, or lessened substrate adhesion by fibrolytic microbes (McAllister et al., 2005; Waghorn, 2008). On the other hand, the linear and quadratic effect observed on VFA, or change in VFA composition (i.e. linear increase in propionate proportion and decrease in acetate:propionate ratio) with higher CT level (Table 5) suggest that a direct effect of CT on rumen methanogenesis is also prevailing. This effect could result from a reduction of available hydrogen, which is a substrate for methanogens (Smith et al., 2005; Tavendale et al., 2005; Bhatta et al., 2009). The reduction in hydrogen availability can be achieved when an alternative metabolic pathway such as propionate production disposes of the hydrogen produced during in vitro fermentation of substrate (López et al., 1999).

The increase in CT level except CT from Rees "A" and CPI63763 reduced total VFA production linearly. Hassanat and Benchaar (2013) also reported a decrease in the in vitro VFA concentration when CT level was increased from 20 to 200 g/kg of DM. However, in the current study, the addition of CT from CPI63767 resulted in less acetate, butyrate and lower acetate:propionate ratio when added at 80 g CT/kg of substrate DM compared with the control and other CT types. This is important in terms of CH_4 reduction, since fermentation of OM to acetate and butyrate produces hydrogen, which is utilized in the rumen to produce CH_4, while substrates that promote production of propionate in the rumen decreases CH_4 production (Tavendale et al., 2005). This strong inverse relationship between propionate and CH_4 production can be predicted from knowledge of interactions among ruminal microbial populations (Morgavi et al., 2010). The extent of linear and quadratic reduction in total VFA production, and the linear increase in propionate proportion and decrease in acetate:propionate ratio for different CT types with increasing CT levels indicates an anti-methanogenic effect, in which the effect depends on the type of CT. Earlier it was reported that the activities of CT on ruminal VFA production and composition vary depending on CT level and source (Beauchemin et al., 2007; Bueno et al., 2008; Hassanat and Benchaar, 2013).

For better assessment of the overall environmental impact of different CT types and levels in ruminant diets other greenhouse gas emissions such as N_2O will also need to be considered in addition to enteric CH_4 production. This is important because CT can also affect protein degradation (Martínez et al., 2006); but they can also increase the utilization of branched–chain VFA (iso-acids) for microbial protein synthesis. In fact, both effects are

likely, as dietary CT have been reported to be associated with reduction of protein degradation in the rumen (Waghorn and Shelton, 1997; Getachew et al., 2008; Hassanat and Benchaar, 2013). The iso-acids VFA arise almost exlusively from the oxidative deamination of branched-chain amino acids such as leucine and isoleucine. In addition, these branched–chain VFA production also decreased linearly as CT level increased and becomes negative when CT were added at higher concentration (Table 5), indicating that there was net uptake of these branched–chain VFA as the result of protection of proteins by CT from rumen deamination. This effect was more pronounced with CT from CPI63767 and added at 80 g CT/kg of substrate or more. In agreement with our results, Makkar et al. (1988) also reported that the protein precipitation capacity of CT depends on the type of tannins and the degree of polymerization. This can have a positive effect by increasing the amount of rumen-escape protein as well as causing a higher flow of microbial proteins to the intestine and hence improve N utilization when CT are supplied in ruminant diets. Moreover, the effect observed on protein fermentation as indirectly evidenced by a change in VFA composition favouring a shift towards propionate and reduction of branched–chain VFA (Table 5) is consistent with a recent in vivo study on sainfoin of Aufrére et al. (2013), who reported that sainfoin CT generated rumen-escape protein and enabled better utilization in the small intestine and higher N retention as the dietary proportion of sainfoin was increased.

CONCLUSIONS

Condensed tannins obtained from sainfoin accessions are promising for reducing rumen CH_4 production. There were significant differences in the anti-methanogenic activity among the CT extracts, which could be attributed to differences in tannin polymer size (mDP values). These size differences may have affected the ability of tannins to interact with dietary fibre and proteins or microbial cells. A decrease in proteolytic activity as indirectly shown by a change in VFA composition favouring a shift towards propionate and reduction in branched–chain VFA production can be seen as a potential advantage in terms of improving N utilization by ruminants. This study generated preliminary evidence that tannin polymer size is an important factor as far as CH_4 and VFA production are concerned. Next to knowledge about the actual degree of polymerization, its proportional distributions could be of interest. A study with a wider range in CT structure (PC:PD and *cis:trans* ratios) is recommended to unambiguously assess the impact of CT structures on activity without detrimental effects on fibre degradation.

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CHAPTER 8

General discussion

INTRODUCTION

Feeds and feeding management strategies have been proposed and evaluated to mitigate CH_4 emissions from dairy cows (Hristov et al., 2013; Knapp et al., 2014). Implementation of these strategies can be variable as many interacting factors can arise depending on the dairy production systems. It is generally acknowledged that improving the quality of carbohydrate and its rumen fermentation characteristics alter ruminal and postruminal digestion (Nocek and Tamminga, 1991; Gozho and Mutsvangwa, 2008; Ferraretto and Shaver, 2015), although quantitative evidence supporting a reduction in enteric methane (CH_4) emission intensity (CH_4 Ei; CH_4 per unit of animal product) in dairy cattle is still limited and requires further investigation. Besides, feeds and feeding management is a very broad area, with many opportunities still available to investigate as mitigation strategy of enteric CH_4 emission. As such, feeding strategies are considered as the most viable strategy that can have effects in the short- to medium-term of reducing enteric CH_4 emissions from dairy production and contribute to the efforts of governments to meet climate change obligations to reduce this greenhouse gas (GHG).

In this thesis, the results presented are of three major feeding strategies to quantify enteric CH_4 emissions from dairy cows and identify opportunities to mitigate the emission of this GHG. The first research category focused on source and quantity of starch in the diet (**Chapter 2** and **Chapter 4**). Furthermore, in the initial phase of the research, it was hypothesized that an in vitro study as a stand-alone approach is not adequate to fully evaluate the potential of feeds and rumen fermentation modifiers to reduce CH_4, because in vitro studies are frequently performed separately rather than in parallel with in vivo studies. Therefore, an experiment comparing the relationship between in vitro and in vivo CH_4 production of dairy cows measured simultaneously under the same conditions was also conducted under this research category and presented in **Chapter 3**. The second research category focused on investigation of the impact of improving quality of grass silage through grassland management (**Chapter 5**). Management strategies evaluated were level of N fertilisation and maturity of grass at harvest, as well as their interaction. The third research category included screening of condensed tannins containing plants (**Chapter 6**) and characterization of condensed tannin extracts obtained from these plants (**Chapter 7**) using an in vitro technique. Results obtained from these studies (**Chapter 6** and **Chapter 7**) can be used to design diets with plants containing this compound in the future, and to investigate under in vivo conditions with regard to the impact on enteric CH_4 emissions.

In the following sections, the work described in this thesis for mitigating enteric CH_4 emissions will be discussed. For ease of discussion, the experiment described in Chapter 3 will be discussed under the third research category. Results are discussed in context of possible practical applicability and in the perspective of broader scope of implications. Finally, implications are provided for future research or possible application of the feeding strategies investigated in this thesis to mitigate enteric CH_4 emissions from dairy cows. This chapter ends with a summary of the main conclusions of this thesis.

1. Dietary starch sources

Increasing ruminally available energy concentration of diets for dairy cows has the potential to enhance milk production through increased metabolizable nutrient supply (Gozho and Mutsvangwa, 2008). Feeding high-producing dairy cows aiming to maximize milk production is often achieved by feeding diets with high starch content. Starch in dairy rations is mainly supplied by concentrate and roughages such as maize silage. However, the quantity of starch included in the ration of dairy cows varies from place to place depending on the availability of grain sources which could affect the rumen fermentation characteristics of the cereal grains used (Herrerasaldana et al., 1990; Huntington, 1997) and on the type of cereal crops and agronomic management applied to grow and harvest these crops for making silage (Johnson et al., 2002a, b). Starch concentration and ruminal degradation characteristics of starch in the maize silage, for instance, depend on the stage of maturity of maize plant at harvest (Johnson et al., 1999). The hypothesis tested here was that inclusion of a high level of starch in the concentrate in the diet (**Chapter 2**) and increasing the starch content of maize silage by increasing maturity of whole-plant maize at harvest (**Chapter 4**), gives rise to a high proportion of the glucogenic volatile fatty acids (VFA), propionate, when fed to dairy cows. In addition, since the site of starch digestion determines the amount of energy available to dairy cows (Reynolds et al., 1997), inclusion of slowly fermentable starch was hypothesized to promote postruminal digestion. The passage rate of substrate was reported to explain 28% of variation in an animal's CH_4 production (Okine et al., 1989). As the fractional rate of starch degradation decreases relative to its rate of passage, the extent of microbial access to the starch is reduced, in turn reducing its ruminal fermentation and CH_4 production.

Type and quantity of starch in the concentrate

In **Chapter 2**, the results presented are from a large animal experiment conducted to investigate the effects of quantity and characteristics of starch in the concentrate on CH_4

emissions of dairy cows. Maize starch was used as a starch source containing a considerable fraction that is resistant to rumen fermentation (Huntington, 1997). To make the starch more rapidly fermentable, technological treatment was applied to the maize grain. Furthermore, it was expected that milk yield would increase when the dietary proportion of starch in the concentrate increased because concentrates generally provide more digestible nutrients than roughage (Hristov et al., 2013), thereby increasing animal productivity. However, in the present study no significant differences in milk production were observed between cows fed diets based on concentrate composed of a high compared to a low level of starch. Fat- and protein-corrected milk (FPCM) yield tended to be lower (–1.5 kg/d) for cows fed diets based on a high level of starch (**Chapter 2**, Table 6).

In contrast, Huhtanen and Hetta (2012) reported a highly significant and positive relationship between dietary concentrate intake and production of milk, and milk fat and milk protein using a meta-analysis approach. Responses of lactating cows to different cereal grains depend on the level of dietary inclusion, the basal ration, physical processing of the cereal grains, the composition of a given batch of cereal grain, and the level of dietary intake (Khorasani et al., 2001). The tendency of higher FPCM in cows fed the low level starch-based diets in the present study might be due to higher dry matter intake (DMI). Furthermore, the results from the present study did not show significant differences in CH_4 Ei and CH_4 yield (g CH_4/kg of DMI); although CH_4 emission per unit of rumen fermentable organic matter (OM) was lower with elevated proportions of both types of starch in the concentrate. This suggests that no abatement with the high level of starch in the concentrate was achieved in the present study, or it was not large enough or not a feasible strategy to reduce enteric CH_4 emissions in dairy cows fed grass silage-based diets. The advantage of feeding starch in reducing enteric CH_4 emission could be higher if concentrates with the high level of starch were not buffered with bicarbonate, or the level of starch included would be increased to a higher level.

To explore the data further, the linear regression analysis based on treatment means of CH_4 emission expressed in terms of unit of FPCM and per DMI were regressed against the level of starch in the diet, and showed tendency to lower CH_4 Ei and CH_4 per unit of DMI (CH_4 yield) with increasing dietary proportion of starch, which agreed with other studies (Lovett et al., 2003; Ellis et al., 2007). Based on the data in **Chapter 2** (Table 7), a linear relationship between CH_4 emissions (CH_4 Ei and CH_4 yield) and dietary starch content were established.

$$CH_4 \text{ Ei (g } CH_4\text{/kg of FPCM)} = 16.27 - 0.0040 \times \text{dietary starch content (g/kg of DM)}$$

CH_4 yield (g CH_4/kg of DMI) = 23.19 – 0.0077 × dietary starch content (g/kg of DM)

The dietary starch in this prediction equation is completely supplied by concentrate. These CH_4 emission predictions showed a negative linear relationship with the proportion of starch in the concentrate. The negative slope indicates a decrease of 0.0040 and 0.0077 g of CH_4 Ei and CH_4 yield, respectively, could occur for every 10 gram starch increase per kilogram of concentrate. Pirondini et al. (2015; **Figure 1**) observed a trend for lower CH_4 Ei (g CH_4/kg of milk) with the high (280 g/kg of DM) compared to the low (238 g/kg of DM) level of starch in the diet, whereas CH_4 Ei (g CH_4/kg of energy corrected milk; ECM) was not affected. In contrast, a more pronounced and linear reduction of CH_4 Ei (g CH_4/kg of ECM, and g CH_4/kg of milk) and CH_4 yield in dairy cows fed diets with low forage to concentrate ratio (starch content of 290 g/kg of DM) compared to high forage to concentrate ratio (starch content of 200 g/kg of DM) has been reported (Aguerre et al., 2011; **Figure 1**).

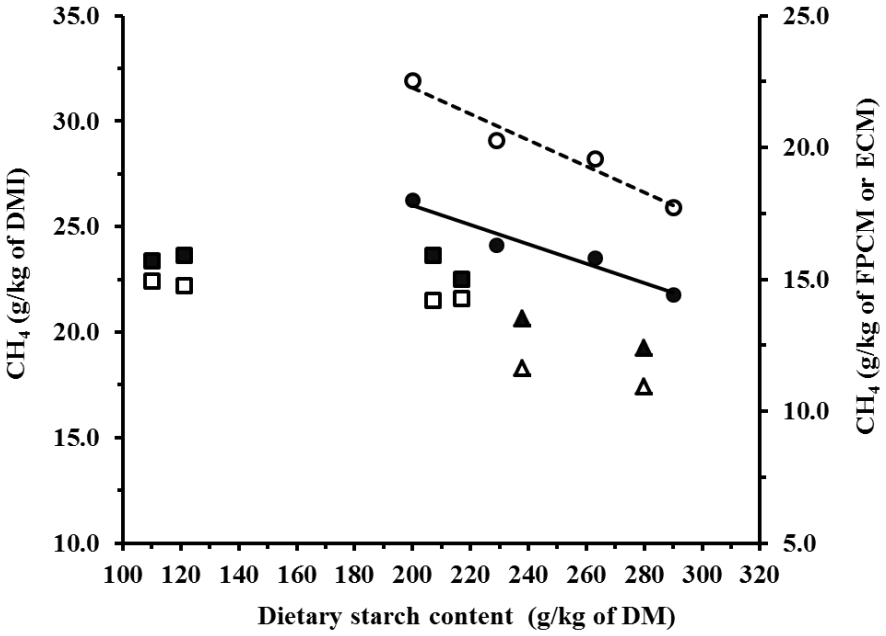

Figure 1. Effects of dietary starch content on CH_4 emissions of dairy cows (g CH_4/kg of FPCM or ECM, closed symbols; g CH_4/kg of DMI, open symbols).
Sources of data: **Chapter 2** (Hatew et al., 2015a) of this thesis (square); Pirondini et al., 2015 (triangle); and Augerre et al., 2011 (circle).

Using a modelling approach, Bannink et al. (2008) also showed that increasing the highly fermentable starch sources in total mixed rations lowers the proportion of feed energy converted to CH_4 by decreasing the acetate:propionate ratio in the rumen fluid. This acetate:propionate can be expected in particular with concentrate-rich diets. In a recent

review, Hristov et al. (2013) concluded that the feeding of starch-rich concentrate is more effective in reducing the amount of energy lost to CH_4 production when diet grain level is beyond 350 to 400 g/kg (DM basis). This implies that for cereal grains such as barley, maize, wheat and sorghum containing about 580 to 770 g starch/kg of DM (Huntington, 1997), inclusion of 350 to 400 g grain/kg could supply 203 to 308 g starch/kg of dietary DM. Results from other studies (Augerre et al., 2011; Pirondini et al., 2015) and shown in **Figure 1** also found that when dietary starch content is greater than 229 g/kg of DM, CH_4 Ei is reduced or showed a tendency to reduce. This indicates that more moderate variation in dietary starch proportion as investigated in **Chapter 2** and buffered with bicarbonate is unlikely to affect CH_4 Ei.

A more pronounced reduction in enteric CH_4 production may occur with starch content in concentrate increased beyond the highest level (217 g/kg of DM) evaluated in **Chapter 2**. Part of such a reduction may be associated with the negative effects of starch fermentation on fibre digestibility as observed in the study of Pirondini et al. (2015). In cows fed high proportions of rapidly fermentable starch, VFA may accumulate in the rumen and cause a decline in pH (Dijkstra et al., 2012), inhibiting fibrolytic and protozoal activity (Dijkstra et al., 1994) and hence a decline in growth of methanogens (Hegarty, 1999) as well as a shift to formation of propionic acid rather than other VFA (Bannink et al., 2008), acting as a sink for hydrogen and reducing CH_4 production. Feeding lactating dairy cows with 720 versus 520 g concentrate/kg of DM (containing 296 vs. 213 g starch/kg of DM) also resulted in a 44% reduction in acetate:propionate ratio and a 59% increase in ruminal propionate concentration, but accompanied by milk fat and total-tract apparent NDF digestibility reduction (Agle et al., 2010). Similarly, a recent meta-analysis of 102 studies with lactating dairy cows showed that increasing diet starch concentrations (average 270 g/kg of DM) increased milk yield but decreased milk fat content, ruminal and total NDF digestibility (Ferraretto et al., 2013). In addition, upon increasing the proportion of concentrate above certain levels may decrease total-tract OM digestibility, which may result in increased amounts of fermentable OM in the manure (Lee et al., 2012) and likely increased CH_4 emissions from stored manure. These trade-off emissions are not desirable (Dijkstra et al., 2011) though not examined in the present study.

Looking at a wider perspective, global consumption of meat and milk products is projected to be more than double by 2050 compared to 2000 (FAO, 2006) and on the other hand demand for cereal grains for human consumption is also projected to increase (World

Bank, 2008). This may raise ethical questions of feeding grain to ruminants for milk and meat production in terms of resource use efficiency and associated environmental effects. Though much of the cereal grain fed to animals is not human grade type (milling quality), in the future the use of more grains in cow rations may be limited because of the possible competition between cereal grain use for milk production by animals and for human consumption. When human grade cereal grains are produced, supply and demand are the main determinant factors for the amount of grain to be fed to dairy cows. For instance, in developing countries where cereal grain demand for human consumption is much higher than the supply (FAO, 2002), the use of grain for dairy rations to minimize CH_4 emission is unforeseen. In contrast, in developed countries where the demand and supply of cereal grain are more balanced or supply exceeds demand for human consumption, the price of grain will also rules whether in any particular condition grains can only be used for human consumption or animal feed (FAO, 2012). A great advantage of keeping ruminants is their ability to convert human inedible and indigestible fibrous feedstuffs into high quality foods (milk and meat) for human consumption. Therefore, applicability of grain feeding to reduce CH_4 would not be simple to implement.

Quantity and quality of starch in maize silage

As discussed above, the scope of increasing grain feeding to dairy cows is limited. Feeding high-quality starch-rich forage such as maize silage can be an alternative source of starch, and could be a better approach to be adopted by dairy producers to reduce enteric CH_4 emissions. The popularity of silages in modern farming systems has increased because of the low production costs and high nutritional value (Wilkinson and Hill, 2003; Khan et al., 2015). For instance, in the Netherlands maize silage constitutes, on average, 25% of the total diet DM of dairy cow ration (Bannink et al., 2011). However, the nutritional value of maize plants depends on the physiological status of plant at harvest (i.e., maturity). The starch content of maize silages varies from 17 to 41% depending mainly on plant maturity at harvest, that is, the proportion of grain in the plant (Noziere et al., 2010). As the maize plant matures, the maize grain kernel undergoes several changes including reduced moisture concentration, increased vitreousness, and increasing encapsulation of starch granules by a protein matrix (Johnson et al., 1999).

In **Chapter 4**, it was hypothesised that increasing maturity of maize at harvest modifies the nutritional composition (increased starch content and a reduced fractional rate of ruminal degradability of starch, and decreased fibre content). Such changes in composition were

expected to decrease CH_4 Ei of cows fed silage made from whole-plant maize harvested at a late compared with an early stage of maturity, and this formed the basis for the design of this experiment. In order to have contrasting maize silages, the whole-plant maize was harvested at a very early (25.1% DM), early (27.7% DM), medium (32.0% DM) and late (40.3% DM) stage of maturity. From a practical point of view, it is not common (at least in the Netherlands) to harvest maize crop at a very early stage of maturity. This may cause problems with ensiling or conservation and also may cause losses of silage DM yield. Harvesting at a very late stage of maturity may also increase risks of contamination with *Fusarium mycotoxin* in the field and may increase aerobic spoilage by yeasts and moulds during storage. Currently, in most parts of the world the recommended practice is to harvest at DM content between 30.0 and 35.0% (Khan et al. 2015). In the Netherlands, for example, the average starch content of maize silage is between 305 and 356 g starch/kg of DM (Bannink et al., 2011), which is similar to the whole-plant maize harvested between the early and a medium stage of maturity in the present study. This shows that the starch content of the maize silage can be increased by simply increasing harvest maturity of the maize crop with minimal or without extra cost and agrees with other studies (**Figure 2**).

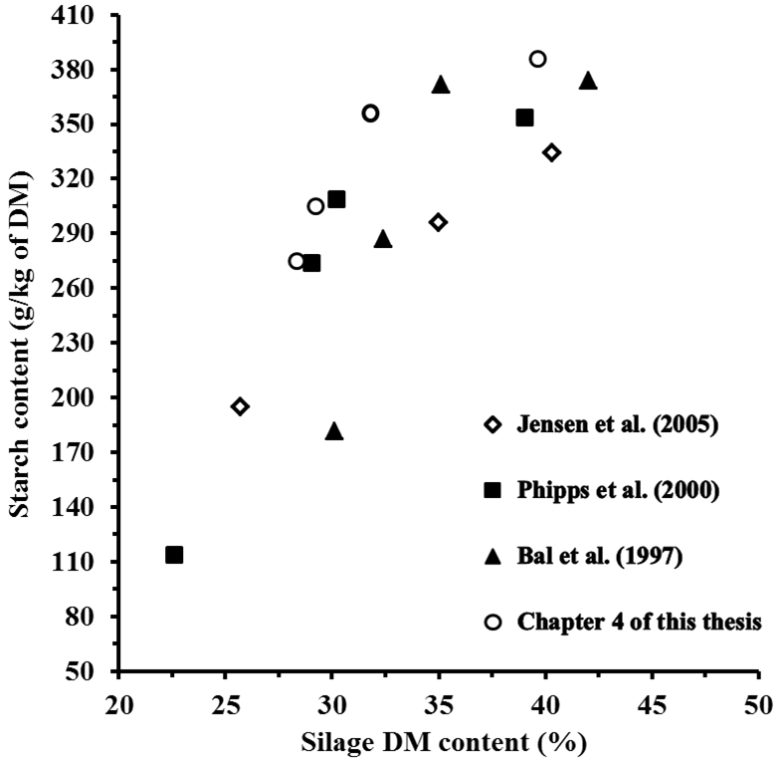

Figure 2. Relationship between DM content and starch concentration of maize silages made from maize harvested at different stages of maturity. Hybrid and growing conditions varied among experiments.

Results from studies investigating the utilization of starch from maize silage is often inconclusive because effects are diluted by the starch provided by the grain in the concentrate, or the two starch sources (i.e. starch in the concentrate and starch from maize silage) could counteract opposite extremes in digestibility to normalize actual cows' performance. In the present study, however, this confounding effect was avoided by including a high proportion of maize silage as the sole source of starch in the diet (75% of total diet DM). It is encouraging that the effects of increasing harvest maturity of maize to mitigate CH_4 emission from dairy cows were positive and can possibly be applied directly in practice by dairy farmers who already practicing harvesting of maize at a lower DM content. It is also important to point out that the milk yield obtained with a diet composed of 75% maize silage of high nutritional value but medium protein content (144 g/kg of DM; **Chapter 4**, Table 5) were obtained by restrict feeding to avoid the confounding effect of feed intake on CH_4 emission. Using the same strategy at ad libitum feed intake may result in more milk yield.

Based on data in **Chapter 4**, a 10 g/kg increase in the DM content of whole-plant maize at harvest reduces CH_4 Ei and CH_4 yield by 0.0071 and 0.0147 g/kg, respectively, and CH_4 emissions can be predicted as:

CH_4 Ei (g CH_4 /kg of FPCM) = 15.139 – 0.0071 × DM content of maize at harvest (g/kg)

CH_4 yield (g CH_4 /kg of DMI) = 26.062 – 0.0147 × DM content of maize at harvest (g/kg)

Methane yield linearly decreased and CH_4 Ei tended to decrease with increasing harvest maturity (**Figure 3**). In agreement with this, Van Gastelen et al. (2015) also found a linear reduction in CH_4 yield and quadratic response in CH_4 Ei (g CH_4/kg of FPCM) in lactating dairy cows fed diets where grass silage was partly or fully replaced with maize silage. Similarly, a quadratic response in CH_4 Ei (g CH_4/kg of ECM) and CH_4 yield of cows offered 100% maize silage compared with those offered 0 and 50% maize silage (maize silage replaced alfalfa silage) where the total mixed ration is composed of forage:concentrate ratio of 60:40 was recorded (Hassanat et al., 2013).

In contrast, with maize silage in partial replacement of barley silage, Benchaar et al. (2014) observed a linear reduction in CH_4 yield as dietary starch content increased from 166 to 256 g/kg of DM, but CH_4 Ei (g CH_4/kg of ECM) was not affected by starch content. Methane Ei (g CH_4/kg of FPCM) observed in the present study (**Chapter 4**) was lower compared to other studies (Hassanat et al., 2013; Benchaar et al., 2014; Van Gastelen et al., 2015). This difference can be due to the difference in the proportion of maize silage included in the diet or difference in the starch content of the silages. Our dietary treatments contained

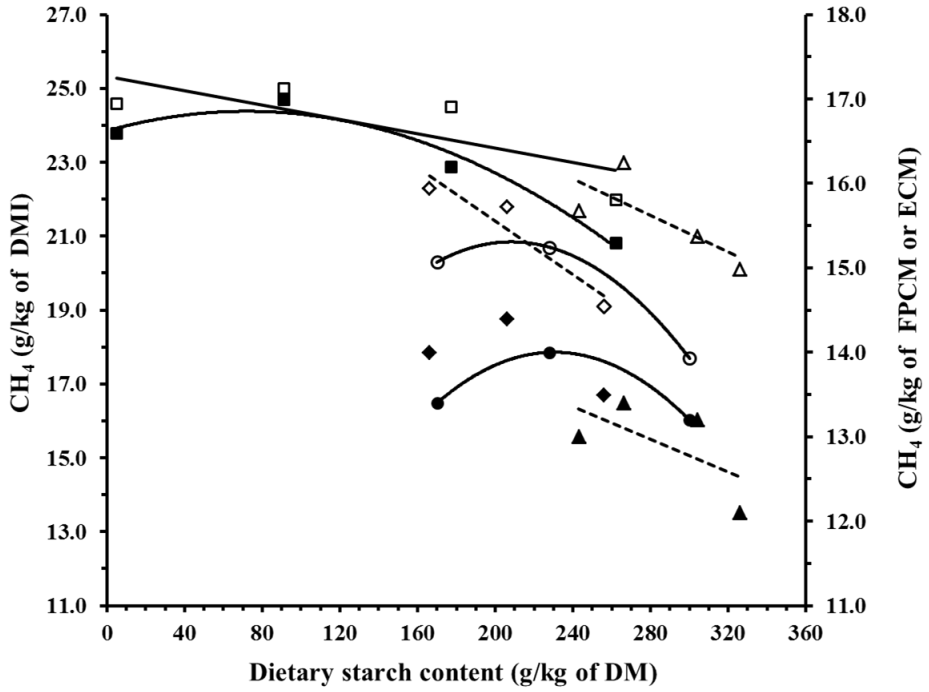

Figure 3. Effects of dietary starch content mainly supplied by roughage on enteric CH_4 production of dairy cows (g CH_4/kg of FPCM or ECM, closed symbols; g CH_4/kg of DMI, open symbols). Source of data: **Chapter 4** of this thesis (triangle); van Gastelen et al., 2015 (square); Benchaar et al., 2014 (diamond); and Hassanat et al., 2013 (circle).

relative more maize silage (750 g/kg, DM basis) versus a maximum of 564 g/kg of dietary DM (Hassanat et al., 2013), and a maximum of 544 g/kg of dietary DM (Benchaar et al., 2014) supplying more starch, resulting in lower CH_4 Ei. In the case of Van Gastelen et al. (2015) although the diet contained up to 800 g/kg of maize silage (DM basis), the starch content of that maize silage was in between maize silage made from maize harvested at a DM content of 27.7 and 32.0% and used in the present study. In addition, in the study of Hassanat et al. (2013) and Benchaar et al. (2014), cows were fed ad libitum compared to restrict feeding in our study, thus the effect of dietary starch might be confounded by DMI. The results reported here suggests possibilities to reduce enteric CH_4 emissions from dairy cows fed maize silage made from whole-plant maize harvested at mature stage of maturity than the current practice used by farmers.

2. Improving quality of grass silage

The effect of maturity of different grass species at cutting on animal production and OM ruminal degradation has been a subject of many studies (Cherney and Cherney, 1997; Waramit et al., 2012). It is well established that animal production decreases as the quality of

forage is decreased by the proceeding development of the plants during growth (Castle and Watson, 1982; Steen, 1992b). This can substantially attributed to decreased digestibility and intake of poor quality forages, mainly because of changes in chemical and physical properties of the plants. In line with this, as ryegrass matures most of its nutritional composition has changed (**Chapter 5**, Table 1) with N and fibre contents mainly affected by N fertilisation rate and stage of maturity of grass at cutting. As the plant matures, the proportion of cell walls increases in the plant material and the increased lignin content is correlated with reduced digestibility of the cell wall material (Jung, 1989; **Chapter 5**, Table 4). In addition, as reported by Heeren et al. (2014), the effective rumen degradability (ERD) of NDF and N of the same grass silages used in the current study (**Chapter 5**) has changed much. The ERD of NDF decreased with maturity and this reduction in ERD of NDF upon an increase in maturity was more pronounced with high than with low N fertilisation. A similar effect was found for ERD of N, although the effects of maturity appeared to be larger with low than with high N fertilisation. These change in rumen degradation characteristics are important for understanding of digestibility and balance of N for cows fed diets based on ryegrass silage. Decreased digestibility leads to lower energy and N contents, providing fewer nutrients to the animal, which, together with the low intake potential, may cause suboptimal feed intake (**Chapter 5**, Table 2).

High OM digestibility of silages made from grass cut at an early stage of maturity is necessary when a high animal production level is to be achieved with forage-based diets. Despite the fact that the low N content in mature forage plants may limit animal production, the efficiency of N capture in the rumen of N-rich silages harvested at an early stage of growth was earlier reported to be poor (Bosch et al., 1992). The faster and more extensive fermentation of early-cut silages in the rumen was expected to lower ruminal pH as observed in previous studies (McAllan et al., 1994; Rinne et al., 1997), which inhibits the growth of cell wall degrading bacteria favouring a higher propionate relative to acetate and butyrate production. The detrimental effect of pH on fibre digestion when early-cut silages are fed however was not observed in the present study. A higher digestibility might be associated with acceleration of rumen passage, which, due to a shorter retention time in the rumen reduces cell wall degradation (Kuoppala et al., 2010) and hence might reduce CH_4 production. However, comparative studies that focussed on the effects of the grass silage quality on CH_4 production by dairy cows are limited. Some modelling approaches predicted that decreased

maturity and increased N fertilisation decreases CH_4 Ei and CH_4 yield (Bannink et al., 2010; Chagunda et al., 2010).

Similar to the low CH_4 Ei and CH_4 yield observed upon feeding maize silage from mature maize at harvest (**Chapter 4**, Table 7), grass silage made from young grass also showed a significant reduction in the CH_4 Ei and CH_4 yield (**Chapter 5**, Table 6). The data in this thesis shows that maturity at harvest is a more important factor than N fertilisation level tested in the present study in affecting grass silage quality including decreased digestibility of OM and crude protein (CP), and increased NDF concentration (**Chapter 5**, Table 1 and Table 4) with advanced maturity which is in line with other studies (Steen and McIlmoyle, 1982; Rinne et al., 2002). The major determinants of the whole-plant nutritive value is the increase in the proportion of the low quality stems with advanced maturity combined with more rapid decrease in the nutritive value of stems compared to leaves. High N fertilisation compared to low fertilisation has improved the CP content and intake by 47 and 18%, respectively. Depending on the rate of N application, N results either in a positive effect by increasing the production of new tissues or in a negative effect by increasing the rate of senescence. The decrease in N content with increased maturity is likely attributed to N translocation from aboveground plant parts (biomass) to below ground organs. Plants use this translocated N for producing new growth the following season. Therefore, delaying harvest maturity considerably decreases forage quality of ryegrass associated with poor animal performance and hence increase CH_4 Ei. In early maturity, not only a lowered cell wall fraction (**Chapter 5**, Table 1) but also an increased OM digestibility (**Chapter 5**, Table 4) and decreased NDF rumen degradability (Heeren et al., 2014) have potentially reduced CH_4 Ei and CH_4 yield.

In practice, the results obtained in **Chapter 5** would influence results of any mitigation exercise and shows that dairy production systems should account for the variation in quality of forage. This assists farmers who produce considerable amounts of high-quality silage to increase milk production and yet contribute to the efforts to mitigate CH_4 emission from dairy production, because their CH_4 Ei will be low. The difference in CH_4 Ei of cows fed the lowest (made from early cut grass) and highest (made from late cut grass) quality silage was as high as 31%, and the difference between the extreme quality (early maturity and low N vs. late maturity and high N) even becomes larger (36%) (**Chapter 5**, Table 6). In line with this, feeding of rye-grass herbage harvested at early stage maturity reduced CH_4 Ei (Warner et al., 2015). Improving grass silage digestibility hence shows the potential to significantly reduce enteric CH_4 Ei and may be a cost effective strategy. Moreover, the interaction effect of

maturity of ryegrass with N fertilisation level on CH_4 production expressed per unit of digestible OM intake (**Chapter 5**, Table 6) suggests agronomic factors such as maturity of cutting and N fertilisation need to be considered when making grassland management decision to reduce CH_4 emission from dairy production. In a broader perspective and in line with our results, Van Middelaar et al. (2014) evaluated the GHG emissions from dairy farming at the chain level (i.e., from production of farm inputs to the farm gate) and compared three feeding strategies, i.e., dietary supplementation of extruded linseed product, supplementation of nitrate, and reducing the maturity stage of grass for grass silage, using life cycle assessment. Using this approach, the authors calculated that reducing the maturity of grass for grass silage was the most cost-effective and most promising strategy to reduce GHG emissions per unit of FPCM.

3. Use of condensed tannin containing plants

In addition to the regular feed (concentrate, maize silage and grass silage) fed to dairy cows on a daily basis, condensed tannins (CT) of various origins have been shown to mitigate enteric CH_4 emission when fed to ruminants as tannin-containing forages (Woodward et al., 2001; Puchala et al., 2005) or as CT extracts fed as dietary additives in vivo (Beauchemin et al., 2007; Bhatta et al., 2013). However, plants produce an enormous range of different tannin types and concentrations (Mueller-Harvey, 2006). The third category of research in this thesis therefore evaluated the effect of variation in CT structural composition in sainfoin accessions on CH_4 production in vitro that would facilitate the use of these compounds as a selection criterion for further in vivo studies. A number of sainfoin accessions (**Chapter 6**) and extracts obtained from selected accessions (**Chapter 7**) were evaluated for their efficacy to reduce CH_4 production using an in vitro gas production technique. The activity of sainfoin CT was found to vary widely depending on variation in its chemical structure. This variation in chemical structure can affect the binding of CT with proteins (microbial and plant proteins) forming tannin-protein complexes via hydrogen bonds, which reduces degradation of protein in the rumen, leading to an enhanced flow of essential amino acids to the small intestine in ruminants fed forages containing CT (Waghorn et al., 1987). This activity could also be important for diets containing excess N, because the protein-binding effect of CT can be beneficial for the environment as reduced N degradation decreases the excretion of N in the urine, the form that is highly volatile (Aufrere et al., 2013). On the other hand, the linear and quadratic effect of CT on VFA, or change in VFA composition (i.e., linear increase in propionate proportion and decrease in acetate:propionate ratio) (**Chapter 7**, Table 5) suggest

that a direct effect of CT on rumen methanogenesis is also prevailing. This effect could be due to a reduction of available hydrogen, which is a substrate for methanogens (Bhatta et al., 2009) and hence reduce CH_4 production. Direct extrapolation of these in vitro results into an in vivo (animal condition) situation, however, might be not feasible at this stage. Because differences in CH_4 production between diets observed in vitro may be considerably larger than those observed in vivo.

As shown in **Chapter 3**, the relationship between in vitro and in vivo CH_4 production (at least for diets based on different types of starch) is moderate ($R^2 = 0.54$) when expressed per unit rumen fermented OM, and even absent when expressed per unit of OM ingested, even when both studies were conducted under the same conditions and measured simultaneously. The results obtained in this study cannot immediately be compared with other studies because of the absence of in vitro – in vivo comparison studies conducted simultaneously. To authors' knowledge, this is the first study where in vitro CH_4 measurements were performed simultaneously with in vivo CH_4 measurement in order to test the potential of an in vitro gas production to predict actual CH_4 production in vivo. The trends are similar to that conducted by Martinez-Fernandez et al. (2013) who showed a proportionally higher reduction in CH_4 production in vitro by two plant compounds by as much as 87 and 96% compared with a relatively lower in vivo CH_4 reduction (33 and 64%, respectively) per unit of DM intake in goats, though in vitro and in vivo experiments were not performed in parallel as done in our study.

In vitro gas production has inherent negative factors, including accumulation of fermentation products, degeneration of the microbial community and disappearance of protozoa, which may affect the results. In addition, the substrates that are used for in vitro incubation are usually ground to fine particles, not representing what the animal is consuming. In principle, the most ideal method to compare in vitro with in vivo CH_4 production of dairy cows ration is by the use of adapted rumen inocula and optimize the use of in vitro gas production techniques through the use of technique that simulate passage of unfermented feed stuffs, continuous supply of substrate and control of pH. With the in vitro technique used in the current studies (**Chapter 3**, **Chapter 6** and **Chapter 7**) continuous supply of substrate, rumen passage and control of pH cannot be simulated, although these factors may be very important to extrapolate the in vitro results into in vivo condition. These limiting factors might have resulted in modest relationship between the in vitro and in vivo CH_4 production when expressed per unit of rumen fermented OM, or the absence of a

relationship when expressed per unit OM intake as well (**Chapter 3**). Furthermore, incubation of the same substrate with rumen inocula obtained from cows adapted to different diets was found to be significantly different in the amount of CH_4 produced per unit of OM incubated (**Chapter 3**, Table 5). This suggests that changing the animal's diet over a longer-term will cause the potential of ruminal microflora to adapt to the diet. Whether the effects observed in the present study are pertinent to other types of ruminant diets and substrates incubated in vitro requires further investigation.

However, it is undisputable that in vitro techniques can be conducted in a short time and used as a rapid screening method for assessment of mitigation options as conducted in **Chapter 6** and **Chapter 7**, used usually for the screening of a large number of treatments with sufficient replication over time, and can be done at a fraction of the cost of an in vivo study. The assessment of the nutritional value and effects of plants containing CT on CH_4 production, therefore, should follow a number of steps as proposed in a previous review (Flachowsky and Lebzien, 2012).

1. Botanical characterization of the plants and their composition
2. Analytical characterization of the active phytogenic substance
3. In vitro studies to test the effects of the substances on rumen fermentation and methanogens (i.e., screening)
4. In vivo studies (feed intake, rumen fermentation and CH_4 emissions) and
5. Long-term feeding studies with target animal species (animal health and performance, quality and safety of food of animal origin, environmental impact, adaptation of microbes).

Accordingly, in **Chapter 6** some of these steps (1 to 3) were performed in collaboration with other institutions involved in the 'HealthyHay' project. The botanical characterization of the sainfoin accessions (step 1) were conducted at National Institute of Agricultural Botany (Cambridge, UK) and results were reported by Hayot Carbonero et al. (2011). Step 2 was performed at Reading University (Stringano et al., 2012), and followed by step 3 at Wageningen University (**Chapter 6** and **Chapter 7**).

As observed by Hatew et al. 2015c (**Chapter 7**), the level of CT required to reduce CH_4 seems considerably higher than the level needed to decrease protein degradation. Thus, care must be taken to ensure that CH_4 suppression using CT does not occur at the expense of fibre digestion, since CT also form complexes with microbial enzymes and interferes with the digestion of fibre (Bae et al., 1993) in addition to binding to microbial and feed proteins. Therefore, when selecting plants containing CT, CT should have a minimal effect on fibre

digestion while maintaining reduction of protein degradation in the rumen. For instance, in the study of Grainger et al. (2009) with dairy cows grazing on pasture and given CT, CH_4 yield was reduced by up to 22%, but milk production, milk fat yield and protein yield were also reduced by 10, 19 and 7%, respectively, whereas CH_4 Ei (g CH_4 per kg of fat and protein yield) was not affected. Moreover, from the limited number of studies available, results of a meta–analysis also indicated that substantial reduction in CH_4 emission is difficult to achieve without decreasing feed digestibility (Goel and Makkar, 2012). The sustained effects of the sainfoin CT as CH_4 reduction strategy, the appropriate in vivo dose, and the effects on the quality of animal products (milk) have not been evaluated in this thesis, all of which are important considerations for large-scale implementation. Therefore, the last two steps (step 4 and step 5) are very important for any study examining rumen fermentation modifiers like CT as a means for lowering CH_4 emissions from dairy cows and require further research.

IMPLICATIONS

During the larger part of the year, dairy cows in the Netherlands are housed and fed a diet, which consists, on average, around 70% forages in a DM. The forage components are mainly consisting of grass herbage, grass silage and maize silage. The degree of variation in the proportion of forage in the total mixed ration can even be wider than this depending on the farm. With the shift in attention towards the contribution of dairy production to CH_4 emissions, dairy farmers can adopt one of the feeding strategies evaluated in this thesis. While increasing starch content in concentrate to very high levels and the use of plants containing condensed tannins examined in this thesis requires further research, other feeding strategies (i.e., improving quality of grass silage, use of maize silage made from maize harvested at late stage of maturity) can be implemented by dairy farmers and are likely to be cost-effective. Harvesting grass at a young age for grass silage is an effective way to improve the diet of the animal to allow them to produce more and reduce CH_4 Ei. This is the most promising strategy that has possibility of being used as an intervention in intensive dairy production systems, because it tends to be low-tech, low-cost, low-risk, and may provide productivity gains. Our results further suggest that increasing the N fertilisation rate in the present range of fertilisation rates and growing conditions of grass was ineffective in reducing enteric CH_4 emission from dairy cows fed this grass silage. Using maize silage made from crops harvested under current practices or at higher maturity to reduce CH_4 emissions as a short- and medium-term strategy, could also be very beneficial.

Even though the in vitro technique has some limitations to represent in vivo conditions, it is useful for the screening of large sets of animal feeds for their use in CH_4 mitigation strategy. However, when collecting rumen inocula for in vitro incubation, it is worthwhile to consider the adaptation of rumen inocula to the type of diets to be investigated in vitro. Because changing animal's diet over a longer-term will cause the ruminal microflora to adapt to the diet and affect the in vitro results. Even when rumen inocula is adapted to the diet caution is necessary when interpreting in vitro results into in vivo as the relationship between them is not high or absent (Hatew et al., 2015b). Whether the relationship between in vitro and in vivo studies observed in the present study are pertinent to other types of ruminant diets incubated in vitro requires further investigation.

GENERAL CONCLUSIONS

- Opportunities to reduce enteric methane emissions from dairy cows were investigated and identified in this thesis. The strategies investigated have possibilities of being adopted in practice by dairy farmers.
- Delaying harvest maturity of maize could be a simple and low-cost measure to increase dietary starch content of maize silage and reduce ruminal rate of degradation of starch, thereby reducing enteric methane emission intensity from dairy farms without negative effects on cow performance.
- Improving grass silage quality by means of increasing organic matter digestibility and lowering cell wall fraction attained by harvesting grass at young stage of maturity reduces methane emission intensity in dairy cows.
- Increasing the level of starch in the diet up to 220 g/kg DM does not reduce methane emission intensity. Increasing the level of starch in the concentrate beyond the level evaluated in this thesis (> 220 g/kg of dietary DM) may reduce enteric methane emissions of dairy cattle, but negative impacts on cow health and fibre digestion may limit its acceptability by farmers. Applicability requires further investigation of the trade-offs of this measure.
- Condensed tannins from sainfoin show potential to reduce in vitro CH_4 production, but require further investigation to fully evaluate their in vivo effects.
- Taken together, results from research work in this thesis show that changes in the basal diet of dairy cows and in roughage production management can substantially reduce the amount of enteric CH_4 produced and affect GHG emission intensity of dairy production.

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SUMMARY (EN)

Summary

Dairy cattle, along with other ruminant livestock, play a significant role in contributing to the world‘s food supply through their ability of converting human indigestible fibrous feeds unsuitable for human consumption to a high-quality protein source (milk and meat). As global demand for animal proteins is expected to rise due to an increase in the human population and consumer income levels, ruminant production faces multiple challenges. These challenges include supply of high-quality foods to meet the projected demand of increasing human population, adaptation to environmental change and, at the same time, reduce impact on the environment. Enteric methane (CH_4) production is the single largest source of greenhouse gas in the dairy sector. Enteric CH_4 production also accounts for an average 6.5% loss of total energy ingested by animal. With such a significant impact, it is imperative to investigate strategies to mitigate enteric CH_4 emission from dairy cows. Although there is no single option to decrease enteric CH_4 emissions from dairy cattle while meeting the growing demand for animal products, feeding strategies are among the most feasible options. This thesis investigated the potential of feeding strategies to mitigate enteric CH_4 production of dairy cows.

In order to investigate the effects of type and level of starch in the concentrate, concentrates that varied in starch rate of fermentation (slowly vs. rapidly rumen fermentable; native vs. gelatinized maize grain) and level of inclusion (low vs. high; 270 vs. 530 g/kg of concentrate dry matter) were formulated and fed to 40 lactating dairy cows in a mixed diet composed of 60:40 (dry matter basis) grass silage to concentrate ratio (**Chapter 2**). The results indicated that inclusion of 530 versus 270 g starch/kg of concentrate significantly reduced CH_4 emission per unit of estimated rumen fermentable organic matter (eRFOM; 43.1 vs. 46.9 g/kg of eRFOM). Furthermore, CH_4 emission per kilogram of eRFOM was higher for slowly compared to rapidly fermentable starch-based diets (47.4 vs. 42.6 g/kg of eRFOM). However, CH_4 expressed per kilogram of fat- and protein-corrected milk (FPCM), per kilogram of DM intake (DMI), or as a fraction of gross energy intake (GEI) was not affected by level or type of starch in the concentrate. A comparison with published results on the effect of starch inclusion in the concentrate indicates that the effect of feeding concentrate starch remained small probably because the level of dietary starch remained too low, or because the diets with the high level of starch were buffered with bicarbonate.

In **Chapter 3** it was hypothesised that in vitro CH_4 measurements for different types and levels of starch in the diet are related to the in vivo CH_4 production if both in vitro and in vivo

CH_4 measurements are performed simultaneously, using the same animals as donor for microbial inoculum when fed (and adapted to) the same dietary material as used as substrate for in vitro incubations. To test this hypothesis, 16 rumen-fistulated lactating dairy cows were used in a complete randomized block design with dietary treatments replicated in four periods. In vitro gas and CH_4 production was measured using an automated gas production system with CH_4 measured at distinct time points. Across the diets tested, 24-h in vitro CH_4 (ml/g of incubated OM) correlated well with in vivo CH_4 expressed per unit of eRFOM (R^2 = 0.54), but not when expressed per unit of OM ingested (R^2 = 0.04). Incubation of the same substrate (grass silage or beet pulp) with rumen inocula obtained from donor cows adapted to different diets produced variable amounts of CH_4 suggesting that it is important to consider the diet of the donor animal when collecting rumen inocula for in vitro incubation. In view of absence or presence of relationships between in vitro and in vivo CH_4 production depending on the unit of expression, this study suggests that complexity associated with rumen fermentation conditions needs to be considered to fully predict in vivo CH_4 production from in vitro measurements.

In **Chapter 4** the effects of feeding whole-plant maize silage (MS) on CH_4 emission of lactating dairy cows was examined using whole-plant maize harvested at a very early (25.1% DM; MS25), early (27.7% DM; MS28), medium (32.0% DM; MS32) and late (40.3% DM; MS40) stage of maturity. A total of 28 lactating dairy cows received one of four dietary treatments, which differed in the type of MS included and designated as T25, T28, T32 and T40 to reflect the DM contents of whole-plant maize at harvest. Treatments consisted of (on DM basis) 75% of MS, 20% concentrate and 5% wheat straw. Starch content of MS increased markedly with maturity (275 vs. 385 g/kg of DM for MS25 vs. MS40). In situ ruminal starch fractional degradation rate decreased linearly from 0.098 to 0.059 /h as maturity increased from MS25 to MS40. Per kg of DMI (21.7, 23.0, 21.0 and 20.1 g/kg of DMI) and as fraction of GEI (6.3, 6.7, 6.3 and 6.0%) CH_4 production decreased linearly with maturity for T25, T28, T32 and T40, respectively. Methane emission intensity (13.0, 13.4, 13.2 and 12.1 g/kg of FPCM) tended to decrease linearly with maturity. Maize silage maturity at harvest did not affect DMI, milk yield, milk contents, energy or N retained and N use efficiency. Increasing the maturity of whole-plant maize at harvest may offer an effective strategy to decrease CH_4 production of dairy cows with feeding MS without negatively affecting cow performance.

In order to explore the effects of N fertilisation of grassland and maturity of grass at cutting on CH_4 emission from dairy cows fed grass silage, two N fertilisation rates (65 vs. 150 kg of N/ha) and three stages of maturity of grass at cutting (early, 28 days of regrowth; mid, 41 days of regrowth; and late, 62 days of regrowth) were investigated (**Chapter 5**). An experiment was conducted with 54 lactating dairy cows that were fed grass silage (mainly ryegrass) and compound feed at a 80:20 ratio (on DM basis). Dry matter intake decreased with N fertilisation and grass maturity, and FPCM production decreased with grass maturity but was not affected by N fertilisation. Apparent total-tract OM digestibility and NDF digestibility decreased with grass maturity but was unaffected by N fertilisation. Methane emission intensity (mean 15.0 ± 1.00 g/kg of FPCM) increased by 31% and CH_4 production per unit digestible OM intake (DOMI) (mean 33.1 ± 0.78 g/kg DOMI) increased by 15% with increasing grass maturity. Methane production per kg DMI (mean 23.5 ± 0.43 g/kg of DMI) and as fraction of GEI (mean 7.0 ± 1.4%) increased by 7 and 9%, respectively, with grass maturity, implying an increased loss of dietary energy with progressing grass maturity. Rate of N fertilisation had no effect on CH_4 emission intensity and CH_4 yield. These results suggest that feeding dairy cows silage of grass cut at an earlier stage of maturity may lead to a reduced CH_4 emission intensity.

In addition to studying the effects of regular feeds (concentrate, maize silage and grass silage) offered to dairy cows on a daily basis, this thesis also examined the effects of condensed tannins (CT) possessing plants and CT extracts on in vitro CH_4 production using a gas production technique. In **Chapter 6** a large number of sainfoin accessions, which were variable in CT content and CT structural composition, and collected from across the world, were screened. Results revealed that substantial variation existed among the sainfoin accessions investigated in terms of their effects on in vitro CH_4 production. This variation was attributed to difference in chemical structure of CT, such as prodelphinidin:procyanidin ratio, rather than to CT content. Promising accessions were selected for further investigation. In **Chapter 7** the effects of types and levels of CT on CH_4 production was examined using four semi-purified CT extracts having contrasting chemical structure and obtained from sainfoin accessions screened in the previous study (Chapter 6). Inclusion of increasing levels of CT linearly reduced CH_4 production, and decreased proteolytic activity as indirectly shown by a change in volatile fatty acids composition towards increased propionate production. This anti-methanogenic activity varied with type of CT and the results indicated that the degree of polymerization of CT is an important factor affecting in vitro CH_4 production and ruminal

protein degradation, which may be caused by the interaction of CT with dietary substrate and/or microorganisms. The level of CT needed to reduce CH_4 seems considerably higher than the level needed to decrease protein degradation, and future in vivo investigations are required to ensure that the suppression of methanogenesis using CT does not occur at the expense of fibre digestion.

Summarizing, delaying the harvest maturity of maize could be a simple and low-cost measure to increase dietary starch content of maize silage and reduce ruminal rate of degradation of starch, thereby offering an effective strategy to decrease CH_4 production in dairy cows without negative effects on cow performance. In addition, improving grass silage quality by cutting grass at a shorter period of regrowth or at a younger stage of maturity reduces CH_4 emission intensity and CH_4 yield. In using the in vitro gas production technique, the complexity related to the rumen fermentation conditions needs to be considered to fully predict in vivo CH_4 production from in vitro measurements. With regard to the use of condensed tannins, the in vitro evidence showed that tannin structure is an important determinant for molar proportion of volatile fatty acids and CH_4 production, but further investigations are required to fully evaluate their effects in vivo. In conclusion, changes in the basal diet of dairy cows and in roughage production management can substantially reduce the amount of enteric CH_4 produced and thereby affect the greenhouse gas emission intensity of dairy production.

SAMENVATTIG (NL)

Melkvee en andere herkauwers die als vee gehouden worden leveren een belangrijke bijdrage aan de wereldvoedselvoorziening vanwege hun capaciteit om vezelrijke grondstoffen, die niet geschikt zijn voor humane consumptie, om te zetten in hoogwaardig eiwit (melk en vlees). Omdar de vraag naar dierlijke producten naar verwachting zal toenemen als gevolg van een toename van de wereldbevolking en inkomensniveau van consumenten, staat de grondgebonden veehouderij voor verschillende uitdagingen. Deze uitdagingen omvatten het voorzien in hoogwaardige voedingsmiddelen voor een groeiende wereldbevolking, aanpassing aan een veranderingen in de leefomgeving en het tegelijkertijd reduceren van de. Enterische methaan (CH_4)-productie is de grootste bron van broeikasgasemissies in de melkveehouderij. Enterisch CH_4 is daarnaast ook een verlies van gemiddeld 6,5% van de totaal opgenomen energie door het dier. Vanwege de grote impact is het noodzakelijk om te onderzoeken welke strategieën enterische CH_4-productie in melkvee kunnen verlagen. Hoewel er niet een enkele oplossing is voor het verlagen van enterische CH_4-productie bij het tegemoet komen aan de groeiende vraag naar dierlijke producten, vallen voederstrategieën onder de meest haalbare opties. Dit proefschrift onderzoekt het potentieel van voederstrategieën om de enterische CH_4-productie in melkvee te verminderen.

Om de effecten van het type en het niveau van zetmeel in het krachtvoer te onderzoeken werden krachtvoeders die verschillen in zetmeelfermentatiesnelheid (langzaam vs. snel afbreekbaar in de pens; onbehandelde vs. ontsloten mais) en in inclusieniveau van zetmeel in de krachtvoeders (laag vs. hoog; 270 vs. 530 g/kg droge stof in het krachtvoer) geformuleerd en verstrekt aan 40 lacterende melkkoeien in de vorm van een gemengd rantsoen met een 60:40 (op basis van droge stof) verhouding voor kuilgras en krachtvoer (**Hoofdstuk 2**). De resultaten lieten zien dat toevoeging van 530 versus 270 g zetmeel / kg krachtvoer de CH_4-emissie per eenheid geschatte pensfermenteerbaar organische stof (eRFOM) aanzienlijk verminderde (43,1 vs. 46,9 g / kg eRFOM). Bovendien was de CH_4-emissie per kilogram eRFOM hoger voor langzaam-fermenteerbaar dan voor snel-fermenteerbaar zetmeel (47,4 vs. 42,6 g / kg eRFOM). Echter, de CH_4-productie uitgedrukt per kilogram vet- en eiwit-gecorrigeerde melk (FPCM), per kg droge stof opname (DMI), of als een fractie van de bruto energie opname (GEI) werd niet beïnvloed door het inclusieniveau of het type zetmeel in het krachtvoer. Een vergelijking met gepubliceerde resultaten over het effect van zetmeel in het krachtvoer geeft aan dat het effect van het inclusieniveau van zetmeel in krachtvoer relatief

gering was omdat het zetmeelniveau in het totale rantsoen te laag bleef, of omdat dat de rantsoenen met het hogere zetmeelgehalte gebufferd werden met bicarbonaat.

In **Hoofdstuk 3** werd verondersteld dat CH_4-metingen etwaarden in vitro van verschillende typen en niveaus van zetmeel in de voeding zouden zijn gerelateerd aan de in CH_4-meetwaarden in vivo, als zowel in vitro als in vivo CH_4-metingen simultaan zouden worden uitgevoerd met dezelfde dieren als donor voor microbieel inoculum voor in vitro incubatie als voor de in vivo metingen. Daarnaast moeten de dieren aangepast zijn en gevoerd worden met dezelfde rantsoencomponenten die gebruikt worden als substraat voor in vitro incubatie. Om deze hypothese te testen, werden 16 pens gefistuleerde lacterende melkkoeien gebruikt in een proef volgens gerandomiseerd compleet blokontwerp met de voederbehandelingen herhaald in vier perioden. In vitro gas en CH_4-productie werden gemeten met behulp van een geautomatiseerd gasproductie systeem. Methaan werd gemeten op verschillende tijdstippen. De in vitro CH_4-productie uitgedrukt in mL / g geïncubeerd organisch materiaal correleerde wel goed met in vivo meetwaarden voor CH_4 per eenheid eRFOM (R^2 = 0,54), maar niet met de in vivo meetwaarden voor CH_4 per eenheid organische stof opname (R^2 = 0,04). Incubatie van hetzelfde substraat (kuilgras of bietenpulp) met pensvloeistof verkregen van donorkoeien die waren aangepast aan verschillende rantsoenen resulteerde in variabele CH_4-producties. Voorgaande suggereert dat het belangrijk is om de voeding van het donordier in overweging te nemen bij het selecteren van pensvloeistof voor in vitro incubatie. Aangezien het al dan niet aanwezig zijn van relaties tussen in vitro en in vivo CH_4 productie afhankelijk was van de eenheid waarin CH_4-productie werd uitgedrukt, suggereert deze studie dat de complexiteit geassocieerd met pensfermentatieomstandigheden in overweging moet worden genomen bij het voorspellen van in vivo CH_4-productie op basis van in vitro metingen.

In **Hoofdstuk 4** werden de effecten van het voeren van gehele-plant maissilage (MS) op de CH_4-productie in lacterende melkkoeien onderzocht. Hiervoor werd snijmaïs geoogst in een zeer vroeg (25,1% droge stof; MS25), vroeg (27,7% droge stof; MS28), gemiddeld (32,0% droge stof; MS32) en laat (40,3% droge stof; MS40) afrijpingsstadium. Een totaal van 28 lacterende melkkoeien kregen vier voerbehandelingen verstrekt die verschilden in het type MS dat opgenomen werd en aangeduid als T25, T28, T32 en T40 naar de droge inhoud zaak van hele plant maïs bij de oogst weerspiegelen. De behandelingen (op droge stofbasis) uit 75% MS van snijmaïs afkomstig van één van de vier rijpheidsstadia, 20% krachtvoer en 5% tarwestro. Het zetmeelgehalte van de MS nam sterk toe met rijpheidsstadium (275 vs. 385

g / kg droge stof voor MS25 vs. MS40). De in situ fractionele afbraaksnelheid van zetmeel in de pens nam lineair af van 0,098 tot 0,059 /uur (MS25 vs. MS40) met een toename in rijpheid. Per kg DMI (21,7; 23,0; 21,0 en 20,1 g / kg DMI) en als fractie van de bruto energieopname (6,3; 6,7; 6,3 en 6,0%) daalde de CH_4-productie lineair met afrijpingsstadium voor T25, T28, T32 en T40, respectievelijk. Er was een trend voor een lineaire daling van de CH_4-emissie intensiteit (13,0; 13,4; 13,2 en 12,1 g / kg FPCM) met afrijpingsstadium. Afrijpingsstadium van MS had geen invloed op DMI, melkgift, melksamenstelling, energie- en stikstofretentie en de efficiëntie van N-benutting. Het oogsten van MS in een later afrijpingsstadium kan gebruikt worden als effectieve strategie voor het verlagen van CH_4-productie in melkvee zonder negatieve effecten op de koeprestaties.

Om de effecten van N-bemesting en leeftijd van gras bij maaien op de CH_4-productie in melkkoeien te onderzoeken die graskuil gevoerd krijgen (**Hoofdstuk 5**), zijn twee N-bemestingsniveaus (65 vs. 150 kg N / ha) en drie perioden voor hergroei tot het moment van maaien aangehouden (vroeg, 28 dagen hergroei; gemiddeld, 41 dagen hergroei en laat, 62 dagen hergroei). Een proef werd uitgevoerd waarin 54 melkkoeien rantsoen gevoerd kregen dat bestond uit kuilgras (voornamelijk raaigras) en krachtvoer in een verhouding van 80:20 (op droge stofbasis). De droge stof opname nam af met N-bemestingsniveau en met het aantal dagen hergroei, en de FPCM-productie daalde met aantal dagen hergroei maar werd niet beïnvloed door het N-bemestingsniveau. De schijnbare fecale verteerbaarheid van organische stof en NDF namen af met het aantal dagen hergroei maar werden eveneens niet beïnvloed door N-bemestingsniveau. Bij een langere periode van hergroei steeg de intensiteit van CH_4-emissie (gemiddeld 15,0 ± 1,00 g / kg FPCM) met 31% en CH_4-productie per eenheid verteerbare organische stof opname (DOMI) (gemiddeld 33,1 ± 0,78 g / kg DOMI) steeg met 15%. De CH_4-productie per kg DMI (gemiddeld 23,5 ± 0,43 g / kg DMI) en als fractie van de bruto energie opname (gemiddeld 7,0 ± 1.4%) stegen met respectievelijk 7 en 9% bij een toename in de lengte van de hergroeiperiode. De intensiteit van de CH_4-emissie en het CH_4-productieniveau werden niet beïnvloed door verschillen in N-bemestingsniveau. Deze resultaten geven aan dat het verstrekken van kuilgras, dat geoogst is in een vroeger stadium van de groeiperiode, aan melkkoeien leidt tot een significant lagere intensiteit van CH_4-productie.

In aanvulling op de effecten van de reguliere voeding (krachtvoer, snijmaïs en graskuil) dat melkkoeien aangeboden krijgen, zijn in dit proefschrift ook de effecten van planten die gecondenseerde tannines (CT) bevatten en van CT-extracten op in vitro CH_4 productie

onderzocht met behulp van een in vitro gasproductietechniek. **Hoofdstuk 6** beschrijft de screening van een groot aantal Sainfoin accessies die waren verzameld vanuit de gehele wereld, die verschilden in CT-gehalte en CT-structuur. Uit de resultaten bleek dat er aanzienlijke verschillen waren tussen de Sainfoin accessies met betrekking tot hun effect op CH_4-productie in vitro. Deze verschillen werden meer toegeschreven aan verschillen in chemische structuur van CT, zoals gekenmerkt door de verhouding prodelphinidine:procyanidine, dan aan totaal CT-gehalte. De veelbelovende accessies werden geselecteerd voor verder onderzoek. In **Hoofdstuk 7** zijn de effecten van de soorten en niveaus van CT op CH_4-productie onderzocht met behulp van vier semi-gezuiverde CT-extracten met een contrasterende chemische structuur en verkregen uit de veelbelovende accessies die gescreend werden in de voorgaande studie (Hoofdstuk 6). Toevoegen van een toenemende hoeveelheid CT resulteerde in een lineaire afname van de CH_4-productiee en een verminderde proteolytische activiteit, welke op een indirecte wijze werd aangetoond middels een verschuiving van het vluchtige vetzuurprofiel ten gunste van propionzuur. Deze anti-methanogene invloed varieerde per type CT en de resultaten gaven aan dat de polymerisatiegraad van CT een belangrijke bepalende factor was die de vitro CH_4-productie en eiwitafbraak beïnvloedde, mogelijk vanwege een interactie tussen CT en geïncubeerde substraat en / of micro-organismen. Het niveau van CT dat nodig is om de CH_4-productie te verlagen lijkt aanzienlijk hoger dan het niveau dat nodig is om de eiwitafbraak te remmen. Toekomstig in vivo onderzoek is nodig om te bevestigen dat de onderdrukking van de methanogenese met behulp van CT niet ten koste gaat van de vezelvertering.

Samenvattend kan gezegd worden dat het uitstellen van de oogst van snijmaïs een eenvoudige en goedkope maatregel kan zijn om het zetmeelgehalte en de pensbestendigheid van het zetmeel te verhogen, wat daarmee een effectieve strategie oplevert om de CH_4-productie in melkvee te verlagen zonder negatieve gevolgen voor de prestaties van de koe. Daarnaast kan de CH_4 emissie-intensiteit en de CH_4-productie ook gereduceerd worden door het verbeteren van kuilgraskwaliteit door maaien te na een kortere hergroeiperiode of in een jonger groeistadium. Bij het gebruik van de in vitro gasproductietechniek moet de complexiteit gerelateerd aan de pensfermentatieomstandigheden meegenomen worden om in vivo CH_4-productie nauwkeurig te kunnen voorspellen uit de in vitro metingen. Wat betreft het gebruik van gecondenseeerde tanninen om de CH_4-productie te verlagen wijzen de resultaten van in vitro onderzoek erop dat de tanninestructuur een belangrijke bepalende factor is voor het vluchtige vetzuurpatroon en de CH_4-productie. Verder onderzoek is nodig

om de effecten volledig te kunnen beoordelen voor een in vivo situatie. Op basis van dit proefschrift wordt geconcludeerd dat veranderingen in het basisrantsoen van melkkoeien en veranderingen in management van ruwvoerproductie de hoeveelheid enterisch CH_4 die geproduceerd wordt aanzienlijk kunnen verminderen, en daarmee ook de intensiteit van de broeikasgasemissie in de zuivelproductieketen.

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The chicken does not forget the person who plucked its tail feathers during the rainy season (‘Mammaaksa’ Oromoo - Oromo proverb)

It is very hard to find Oromo people make a speech or talk without citing ‘mammaaksa’ (a proverb). ‘Mammaaksa’ can summarize a few number of sentences. Oromo elders spice their talk to pass the right message to audience. That is why I preferred to start my acknowledgements with this classical Oromo proverb. After I hold MSc degree, holding PhD degree was one of my dream. But I would have never imagined that the Netherlands is linked to my destiny. Netherlands has not only granted me the opportunity to better myself in preparation for my future, but has given me the resources and memories that will last forever. When first I joined Wageningen university to work as junior researcher on ‘HealthyHay’ project my ambition was to go for a higher degree. During the first year of my employment I learned a lot about how to work with people of different characters and culture. In addition, due to the nature of this project I got a chance to travel to many European countries which gave me the chance to meet different professionals to share ideas and experiences. On the other hand, to full fill my ambition I have to invest a lot of time and energy in doing my work to which I was assigned. In addition, it required me to have some patience before I started PhD, as Oromo says **“Obsan anaan goromsa dhugan”**. Literally it means it is only with patience that someone will drink milk from a heifer. At the end, my patience has worked out and I started the PhD journey. The encouragement, advice and contribution of Wilbert Pellikaan and other ANU staff members were incredible for my smooth transition from pre-PhD job to PhD journey. Especially the advice of Pellikaan was beyond all my expectations.

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Bayissa Hatew

ABOUT THE AUTHOR

Curriculum vitae

Bayissa Hatew was born on December 4, 1976 and grew up in Gindeberet, Oromia, Ethiopia. He studied secondary school at Gindeberet high school and then after joined Jimma College of agriculture. In August 1992, he graduated in diploma with great distinction (magna cum laude). In 1993, he joined Ethiopian Institute of Agricultural Research (EIAR) and worked until 1997. In 1998, he joined Debub University and obtained BSc degree with distinction (cum laude) in July 2000. In 2001, he was employed as zonal coordinator of Livestock Early Warning System (LEWS) project and worked until 2001. The LEWS project was conducted in five East African countries in collaboration with EIAR, International Livestock Research Institute and Texas A&M University, USA. From January 2002 to September 2005, he worked as assistant researcher at EIAR. In October 2005, Bayissa was granted Pears foundation MSc scholarship and joined The Hebrew University of Jerusalem, Israel. During his MSc study, Bayissa followed specialization in Human Nutrition and obtained MSc degree in March 2008. His MSc thesis was about identification and characterization of antimicrobials agents to be used in food preservation and this thesis resulted in one scientific publication. Between January to September 2008, Bayissa worked at Agricultural Research Organization (ARO), Volcani centre, Israel. From his research work at ARO, Bayissa co-authored one scientific publication. In September 2008, he was awarded with Erasmus Mundus scholarship and joined Catholic University of Leuven, Belgium. In Leuven University, he followed courses and training in Food Science, Technology and Nutrition. In November 2009, he was employed by Wageningen University, the Netherlands, to work as a junior researcher on Marie Curie Research Training Network 'HealthyHay' project. His major research in 'HealthyHay' project focused on effects of variation in chemical structure of condensed tannins on in vitro methane production, and this research resulted in two scientific publications. During the last four years, Bayissa was a full-time PhD researcher at Wageningen University. His PhD research investigated the effect of feeding strategies to mitigate enteric methane production of dairy cows.

List of publications

Peer-reviewed journal articles

Effects of dietary starch content and rate of fermentation on methane production in lactating dairy cows.
Hatew, B., Podesta, S.C., van Laar, H., Pellikaan, W.F., Ellis, J.L., Dijkstra, J., and Bannink, A.
Journal of Dairy Science (2015) 98, 486−499.

Relationship between in vitro and in vivo methane production measured simultaneously with different dietary starch sources and starch levels in dairy cattle.
Hatew, B., Cone, J.W., Pellikaan, W.F., Podesta, S.C., Bannink, A., Hendriks, W.H., and Dijkstra, J.
Animal Feed Science and Technology (2015) 202, 20−31.

Increasing harvest maturity of whole-plant corn silage reduces methane production of lactating cows.
Hatew, B., Bannink, A., van Laar, H., de Jonge, L. H., and Dijkstra, J.
Journal of Dairy Science (2015) (Accepted).

Impact of variation in structure of condensed tannins from sainfoin (*Onobrychis viciifolia*) on in vitro ruminal methane production and fermentation characteristics.
Hatew, B., Pellikaan, W.F., Hendriks, W.H., Stringano, E., Hayot Carbonero, C., Smith, L., and Mueller-Harvey, I.
Journal of Animal Physiology and Animal Nutrition (2015) DOI: 10.1111/jpn.12336.

Diversity of condensed tannin structures affects rumen in vitro methane production in sainfoin (*Onobrychis viciifolia*).
Hatew B., Hayot Carbonero, C., Stringano, E., Sales, L.F., Smith, L.M.J., Mueller-Harvey, I., Hendriks, W.H., and Pellikaan, W.F.
Grass and Forage Science (2014) 70, 474−490.

Effects of nitrogen fertilisation rate and maturity of ryegrass silage on methane emission by lactating dairy cows.
Warner, D., Hatew, B., Klop, G., Podesta, S.C., van Gastelen, S., van Laar, H., Bannink, A., and Dijkstra, J.
Animal (2015) DOI:10.1017/S1751731115001640.

Effect of nitrogen fertilisation rate and regrowth interval of ryegrass herbage on methane emission of zero-grazing lactating dairy cows.
Warner, D., Podesta, S.C., Hatew, B., Klop, G., van Laar, H., Bannink, A., and Dijkstra, J.
Journal of Dairy Science (2015) 98, 3383−3393.

Rumen degradation characteristics of ryegrass herbage and ryegrass silage are affected by interactions between stage of maturity and nitrogen fertilisation rate.
Heeren, J.A.H., Podesta, S.C., Hatew, B., Klop, G., van Laar, H., Bannink, A., Warner, D., de Jonge, L.H., and Dijkstra, J.
Animal Production Science (2014) 54, 1263−1267.

Antagonistic intestinal microflora produces antimicrobial substance inhibitory to *Pseudomonas* species and other spoilage organisms.
Hatew, B., Delessa, T., Zakin, V., and Gollop, N.

Journal of Food Science (2011) 76, M522−530.

Inhibition of aconitase in citrus fruit callus results in a metabolic shift towards amino acid biosynthesis.
Degu, A., Hatew, B., Nunes-Nesi, A., Shlizerman, L., Zur, N., Katz, E., Fernie, A.R., Blumwald, E., and Sadka, A.
Planta (2011) 234, 501−513.

Conference contributions and non-refereed papers

Correspondence between in vitro and in vivo rumen methane production obtained with different starch sources and starch levels.
Hatew, B., Cone, J.W., Pellikaan, W.F., Podesta, S.C., Hendriks, W.H., Bannink, A., and Dijkstra, J.
In: Joint Annual Meeting of ADSA/ASAS/CSAS, 20−24 July 2014, Kansas City, USA.

Effects of type and level of starch in concentrate on methane emission in lactating dairy cows.
Hatew, B., Podesta, S.C., van Laar, H., Pellikaan, W.F., Ellis, J.L., Dijkstra, J., and Bannink, A.
In: Proceedings of the 5th Greenhouse Gases and Animal Agriculture (GGAA) conference, 23−26 June 2013, Dublin, Ireland.

The effect of nitrogen fertilization level and maturity of grass herbage on methane emission in lactating cows.
Podesta, S.C., Hatew, B., Klop, G., van Laar, H., Kinley, R.D., Bannink, A., and Dijkstra, J.
In: Proceedings of the 5th GGAA conference, 23−26 June 2013, Dublin, Ireland.

Effect of adaptation of rumen fluid to starch fermentation on in vitro methane production.
Cone, J.W., Hatew, B., Podesta, S.C., Pellikaan, W.F., Dijkstra, J., & Bannink, A.
In: Proceedings of the 5th GGGAA conference, 23−26 June 2013, Dublin, Ireland.

Effects of condensed tannin extracts from sainfoin (*Onobrychis viciifolia* Scop) on rumen in vitro methane production and fermentation characteristics.
Hatew, B., Pellikaan, W.F., Hendriks, W.H, Mueller-Harvey, I., and Stringano, E. (2011).
In: Proceedings of the 8th International Symposium on the Nutrition of Herbivores, 06−09 September, Aberystwyth, UK.

Effects of condensed tannin extracts from sainfoin (*Onobrychis viciifolia* Scop) on rumen in vitro methane production and fermentation characteristics: A potential source for mitigating methane emission.
Hatew, B., Pellikaan, W.F., Hendriks, W.H., Stringano, E., and Mueller-Harvey, I.
In: 36th Animal Nutrition Research Forum − A platform to present Animal Nutrition Research in Belgium and the Netherlands, 19 April 2011, Leuven, Belgium.

NIR spectroscopy for predicting the nutritional, anthelmintic and environmental effects of sainfoin.
Mueller-Harvey, I, Lorenzo, M.M, Stringano, E., Barnes, R., Oliver, F., Theodoridou, K., Aufrere, J., Hatew, B., Pellikaan, W., Manolaraki, F., Hoste, H., Carbonero, C.H., and Smith, L.
In: Proceedings of the 8th International Symposium on the Nutrition of Herbivores, 06−09 September 2011, Aberystwyth, UK.

Training and supervision plan

Training and supervision		Graduate school
Name of PhD student:	Bayissa Hatew	
Group:	Animal Nutrition	
Daily supervisors:	Dr J. Dijkstra, Dr A. Bannink and Dr W. F. Pellikaan	
Supervisor:	Prof. Dr W. H. Hendriks	

Education and training	Year
The basic package (3 ECTS[1])	
Philosophy and ethics of food science and technology	2014
WIAS introduction course	2012
Scientific exposure (11 ECTS)	
International conferences	
Joint annual meeting of the ADSA/ASAS/CSAS, 20 – 24 July, Kansas City, Missouri, USA.	2014
Greenhouse gases and animal agriculture, 23 – 26 June, Dublin, Ireland.	2013
Dairy cattle nutrition "Feed efficiency 2013", 21 November, Wageningen, the Netherlands.	2013
International symposium on the nutrition of herbivores, 6-9 September, Aberystwyth, UK.	2011
Seminars and workshops	
WIAS science day, Wageningen, the Netherlands.	2015
Developments in ruminant nutrition, Wageningen, the Netherlands.	2013
Nutritional management in early lactation, 25 October, Wageningen, the Netherlands.	2012
Parasitology workshop, 13 – 17 April, Toulouse, France.	2010
Careers workshops – Career planning, job searching strategies and presentations, 16 – 20 November, Reading, UK.	2009
Presentations	
Joint annual meeting of the ADSA/ASAS/CSAS, 20 – 24 July, Kansas City, Missouri, USA (Oral presentation).	2014
Greenhouse gases and animal agriculture, 23 – 26 June, Dublin, Ireland (Oral presentation).	2013
Greenhouse gases and animal agriculture, 23 – 26 June, Dublin, Ireland (Poster presentation).	2013
Greenhouse gases and animal agriculture, 23 – 26 June, Dublin, Ireland (Poster presentation).	2013
International symposium on the nutrition of herbivores, 6 – 9 September, Aberystwyth, UK (Poster presentation).	2011
Animal nutrition research forum, 19 April, Leuven, Belgium (Poster presentation).	2011

In-depth studies (9 ECTS)	
Statistics for the life sciences	2015
Advanced feed evaluation	2013
Fatty acids in dairy cattle in relation to product quality and health	2012
Advanced statistics course: Design of experiments	2011
Analytical work and possibilities within animal nutrition sciences	2009
Statutory courses (3 ECTS)	
Use of laboratory animals (Article 9 authorization)	2011
Professional skills support courses (3 ECTS)	
Data management	2014
Techniques for writing and presenting a scientific paper	2012
Course supervising MSc thesis work	2011
Information literacy including EndNote introduction	2011
Research skills training (6 ECTS)	
Preparing own PhD research proposal	2012
Didactic skills and training (5 ECTS)	
Supervising practicals: Applied animal biology	2014, 2015
Supervising and examine MSc major thesis	2011, 2015
Reviewing research proposals: Master cluster proposals	2011
Total = 40 ECTS	

[1]One ECTS credit equals a study load of 28 hours.

COLOPHON

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